bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

(Subclinical mastitis alters dairy cow behavior)

Subclinical Mastitis in Dairy Cows is Associated with Changes in Salivary Serum Amylase-A, Social Behavior and Activity Profiles

G. Caplen[1*], S. Held[1]

gina.caplen@bristol.ac.uk; suzanne.held@bristol.ac.uk

[1]Animal Welfare and Behaviour Group, Bristol Veterinary School, University of Bristol, Langford, Bristol, BS40 5DU, UK

[*]Corresponding author

ABSTRACT

The relationship between behavior and low-level, subclinical systemic inflammation was investigated in a group of matched-pair (sub-clinical mastitis, **SCM**, versus clinically healthy control, **CTRL**) intensively housed dairy cows (n = 34) over short (24h) distinct periods. We report, for the first time, that an increase in an inflammatory marker (salivary serum amylase-A, **SAA**) occurs during the early stages of a bovine disease. SAA was higher in SCM cows, and positively correlated with somatic cell count, the defining parameter of mastitis. SCM cows were observed to display reduced activity (behavioral transitions and distance moved), and reductions in several measures of social behavior including: social exploration, social reactivity (following the receipt of agonistic behavior), performance of social grooming and head butts, and the receipt of challenges. In addition, SCM cows received more head swipes, and spent a greater proportion of time lying with their head on their flank than CTRL cows. SCM cows also demonstrated a preference for lower-risk 'within-herd feeding'; a greater proportion of time feeding was spent in direct contact with herd-members, and a lower proportion of time feeding was spent at self-locking feed barriers, than the CTRL cows. We also present evidence for diurnal differences in the daily behavioral routine between the two groups: SCM cows appear to shift their activity (social and otherwise) to quieter times of the day, a tactic that could actively avoid agonism. Many behavioral measures were found to correlate with SAA in a direction consistent with predictions for sickness behavior. We conclude that salivary SAA, social behavior and activity changes offer potential for use in the detection and monitoring of pre-clinical inflammatory disease states in cows.

Keywords: Cows; SAA; Sickness; Social behavior; Sub-clinical mastitis; Welfare

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1. INTRODUCTION

Infectious disease is highly detrimental to animal welfare and can negatively impact dairy herd profitability via production losses and expensive treatment costs (Grohn et al., 2003; González et al., 2008). It is imperative that emerging health problems are identified and treated as soon as possible; however, low level inflammatory processes and early symptoms of sickness and disease are not easily recognised in any animal (Weary et al., 2006). Although no abnormalities in the gland or milk are visible during subclinical mastitis in cows, milk production drops, milk somatic cell counts (**SCC**) elevate, and inflammation (with or without an intramammary pathogen) occurs (Sordillo et al., 1997). In cows, effective health monitoring is further hampered by logistical difficulties associated with direct animal observations within large open barn systems, reductions in human interaction linked with the substantial uptake in robotic milking units, and the tendency of cattle (as a prey species) to display only subtle indicators of pain/weakness (Gleerup et al., 2015). Advances in image analysis now allow automated recognition of individual cows within a herd (Andrew et al., 2020), accurate identification of some health-related abnormal behaviors, e.g. foot disease (Gu et al., 2017), and detection of social interactions (Guzhva et al., 2016). The identification of behaviors associated with subclinical disease may therefore find application in future diagnostic software algorithms targeted at early disease monitoring in dairy cows (see e.g. Wagner et al. 2020).

Sickness is an adaptive response to infection and inflammation, characterized by endocrine, autonomic, and behavioral change. Infection triggers the immune system to initiate a febrile response and reprioritise behavior (Hart, 1988; Dantzer et al., 2008). This process is mediated by pro-inflammatory cytokines acting upon the CNS (Dantzer and Kelley, 2007). ‘Sickness behavior’ is one such strategy to facilitate recovery, achieved via a reduction in physical activity (energy conservation) and the minimisation of heat loss. Sickness behaviors are useful for early disease detection (see reviews: Weary et al., 2009; von Keyserlingk and Weary, 2010). They include anorexia and withdrawal from the physical and social environment (Dantzer and Kelley, 2007; Tizard, 2008; Hart, 2010). Behavior in healthy animals can be thought of as being of two types in relation to fitness: ‘core maintenance’ (or ‘high-resilience’) with immediate, short-term benefits (e.g. sleep, feed, drink) and ‘luxury’ (or ‘low-resilience’) with delayed, longer-term benefits (e.g. play, exploration, grooming) (Dawkins, 1990; Špinka et al., 2001; Weary et al. 2009). Due to the relative ease with which core behavior can be automatically monitored in dairy cows this has received most attention

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

to date. Changes in feeding behavior associated with bovine clinical disease, for example, have been used to predict ketosis (Gonzales et al., 2008), respiratory disease (**BRD**) (Toa□-Rosenstein and Tucker, 2018), clinical lameness (Gonzalez et al., 2008), metritis (Schirmann et al., 2016; Neave et al., 2018) and mastitis (Fogsgaard et al., 2012; Sepúlveda-Varas et al., 2014). However, because core behaviors are essential for short-term survival, they will only start to decline at relatively late disease stages (e.g. Littin et al., 2008) and have low sensitivity during the early stages (Sepúlveda-Varas et al., 2014). A change in low-resilience behaviors such as social exploration and interaction, however, could potentially flag disease sooner because their immediate importance is further diminished when the animal's energy resources are diverted to fight infection (Dawkins, 1990). We, therefore, here investigate whether any such changes are detectable in subclinical mastitis.

In cows, serum amyloid A (**SAA**), a non-specific inflammatory marker, primarily synthesised in the liver and readily measured in serum, is a key acute phase protein (**APP**) (Murata et al., 2004). APPs have demonstrated great utility in the identification of infectious disease. Increased SAA levels have been reported in cows with BRD (Joshi et al., 2018), reticuloperitonitis, metritis (Nazifi et al., 2008), and subclinical mastitis (Kovac et al., 2011; Nazifi et al., 2011; Kovacevi-Filipovic et al., 2012). A mammary isoform of SAA, synthesised by infected glands within the udder, is also upregulated in milk during sub-clinical mastitis (Eckersall et al., 2006; Kovac et al., 2011; Kovacevi-Filipovic et al., 2012). Although the main veterinary focus for salivary bioscience has been directed at companion animals (Prickett and Zimmerman, 2010; Cerón, 2019), enormous potential exists for non-invasive APP measurement in farm animal saliva; the presence of SAA in bovine saliva has been confirmed (Lecchi et al., 2012; Rahman et al., 2013). To date, very few studies have linked sickness behavior with physiological measures of inflammation in cattle. Des Roches et al. (2017) report correlations between pain indices (including attitude and attentiveness) and serum SAA prior to, and following, intra-mammary challenge with *E. coli*. A later study, utilising a similar mastitis model, identified lower mood (greater lethargy, dejection and su□ering) in mastitic cows, and this was associated with clinical indicators and high SAA levels (des Roches et al., 2018).

The key aim of the current study was to identify any changes in SAA, social behavior and other behavioral signs of sickness associated with subclinical disease states (here mastitis) in dairy cows; this with a view to supporting early detection of disease and identification of

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

more chronic, subclinical, inflammation states. To this end we: (a) compared the behavior of cows with subclinical mastitis with matched healthy cows and, (b) correlated key behavioral measures with both milk SCC (as a measure of local infection severity) and salivary SAA (as a measure of systemic inflammation). We thus investigate for the first time whether SAA measurement in bovine saliva has the potential to detect systemic inflammation associated with a sub-clinical condition.

2. MATERIALS AND METHODS

2.1. Ethics Statement

The study was conducted between October 2017 and February 2018 at Bristol Veterinary School dairy farm. The experimental procedures were approved by the Animal Welfare and Ethical Review Board at the University of Bristol.

2.2. Animals

Focal cows (n = 34) were part of an indoor commercial Holstein-Friesian dairy herd (n = 200) and resided within the low milk-yield group (approx. n = 80 cows) at the time of the study; having been part of the group for at least one month prior to data collection they were well-established within the social dominance hierarchy. Cows were housed within a free-stall barn; their section contained: 93 lying stalls with sand bedding, three drinking troughs, a swinging brush (DeLaval), automatic floor scrapers and vulcanized rubber floors in the front (feeding) alley. Cows were milked three times daily (the low yielders at 06:00, 14:00 and 22:00h) and fed a total mixed ration once daily (06:00h). The feed passage was accessible via self-locking yokes (n = 42) and an open section of post-and-rail feed barrier. Only clinically healthy non-lame cows (mobility score ≤1; AHDB, 2015) were selected for inclusion within the study. To control for confounding effects (air temperature, reproductive status, parity, and stage of pregnancy) data were collected from cows in matched pairs, whereby each pair comprised one cow with subclinical mastitis (**SCM**) and a clinically healthy control (**CTRL**). Cows with a SCC of >200 (x1000 cells/ml) were classified as SCM (Madouasse et al., 2010), while cows with a SCC of <100 (x1000 cells/ml) were classified as CTRL. The focal group comprised pregnant (n = 24) and nonpregnant (n = 10) cows, primiparous (n = 20) and multiparous (parity of 2: n = 2; 3: n = 2; 4: n = 6; 5: n = 4) individuals, and the time to expected calving date ranged from 55 - 236 days.

2.3. Somatic Cell Count

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Composite quarter milk samples were collected at the second (afternoon) milking on the day prior to behavioral observations (Day 1). Somatic cells were manually counted using a standard direct microscopic methodology (ISO 13366-1, 1997) following staining with Newman-Lampert stain solution: Levowitz-Weber modification (Newman's Stain Solution: modified, 01375, Sigma-Aldrich).

2.4. Behavioral Measures

Each focal cow was fitted with a coloured collar to facilitate recognition (Day 1). Two CCTV systems (N441L1T, Annke®, CA 91748, US), including six cameras were used to record video footage from the entire low-yield pen. Continuous behavioral data were scored retrospectively from video for each focal cow for 24h starting from 00:00h (Day 2); all measures are described in Table 1. The following behaviors were not analysed individually but included within the measure of total behavioral transitions ('Trans'): eat sand, lick salt, paw sand, run, shake head, stand, and walk. Rumination was not logged as it proved difficult to definitively identify from the video footage. Tendencies for/against social proximity were investigated using nearest neighbour scores. When the focal cow was located at the feed barrier or resting within a cubicle the number of other cows (0, 1 or 2) in proximity were recorded. At the feed barrier proximity was defined as physical flank-to-flank contact. In a cubicle it was the occupation of (i.e. another cow recumbent within) an adjoining cubicle. To enable the calculation of distance moved ('**Dist**') the pen floorspace was hypothetically subdivided into 29 units (4.8 m wide); front and back passages were each divided into 13 units (F1-13 and B1-13, respectively), in addition to three raised trough areas (M1, M7, M13, Figure 1). The location of each cow was recorded at 5 min scan intervals and 'Dist' was calculated as the number of units crossed within a specific time-period.

INSERT FIFGURE 1 NERE HERE

Several different data sets were analysed. The main data set (**24h**) comprised all data for the 24h observational period. Behavior associated with fresh feed delivery was examined using 60 min of data collected from each cow immediately following their individual return to the home pen after first milking (**1hPostM1**). Diurnal differences in behavior between the two groups were assessed using hourly blocks of data for specific 'key' measures of interest (**Diurnal**). Data was not available for 06:00, 14:00 or 22:00h as the cows were in the milking parlour or collecting yard during these periods.

UOB Open

171 **Table 1:** Cow behavioral measures used within the study.

Measure	Abbreviation – Definition
(A.) Maintenance	
Lie, s	‘**Lie**’[c]: horizontal resting position, with undercarriage or flank in contact with the floor. Includes 'rising'.
Drink, s	‘**Drink**’[b]: ingestion of water at any of the three troughs.
Feed, s	‘**Feed**’[c]: actively ingest/chew food while at the feed barrier.
Head on flank, s	‘**HOF**’[b]: lying with the head in contact with the flank, pointing backwards towards the rump. Associated with active sleep.
Brush, s	‘**Brush**’[b]: use of the mechanical brush for scratching/grooming.
Comfort, s	‘**Comfort**’[b] = ‘Lick self’ + ‘Rub self’ (rub self upon pen furniture) + ‘Scratch self’ (scratch self, using back foot).
Explore environment, s	‘**ExpEnv**’[c] = ‘Explore food’ (sniff/nose food) + ‘Explore pen’ (sniff/lick any part of the barn) + ‘Explore sand’.
Explore social, s	‘**ExpSoc**’[c] = ‘Explore cow’ (sniff a conspecific - no reciprocation) + ‘Mutual sniff’ (reciprocal sniffing).
(B.1) Social: Agonistic	Each was further classified as being: given (G), received (R), received with displacement (+D). See ‘Body push’ for example.
Body push: give, n	‘**BPG**’[b]: sideways shunt delivered by the flank, designed to displace a conspecific (e.g. to access feed barrier).
Body push: receive, n	‘**BPR**’[b]: receipt of a body push.
Body push: displacement, %	‘BPR+D’: receipt of body push, immediately (≤2 s) prompting the focal cow to be displaced. ‘**%BPR+D**’[a] = (‘BPR+D’/‘BPR‘) x 100
Challenge	‘**CG**’[b]: perform a threatening non-contact gesture, aimed at displacing a conspecific (e.g. short-charging, determinedly approaching, or facing/staring at a conspecific with head lowered). [‘**CR**’[b], ‘CR+D’, ‘**%CR+D**’[a]]
Head butt	‘**HBG**’[b]: violently striking a conspecific (on the body or head) using the front of the lowered head, without reciprocation. [‘**HBR**’[b], ‘HBR+D’, ‘**%HBR+D**’[a]]
Head push	‘**HPG**’[b]: using the head to gently push a conspecific's head/body. Usually observed as prolonged/sustained contact. [‘**HPR**’[b], ‘HPR+D’, ‘**%HPR+D**’[a]]
Head swipe	‘**HSG**’[b]: sideways swipe of the head often directed at a conspecific's head. Usually observed at the feed barrier. [‘**HSR**’[b], ‘HSR+D’, ‘**%HSR+D**’[a]]
Focal social: give, n	‘**FocSocG**’[c] = ‘BPG’ + ‘CG’ + ‘HBG’ + ‘HPG’ + ’HSG’

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Focal social: receive, n	'**FocSocR**'[c] = 'BPR' + 'CR' + 'HBR' + 'HPR' + 'HSR'
Focal social: displacement, %	'**%FocSocR+D**'[a] = [('BPR+D' + 'CR+D' + 'HBR+D' + 'HPR+D' + HSR+D')/'FocSocR'] x 100
Mutual head butt, n/s	'**MutHdButt**'[b]: mutual head butting between the focal cow and a conspecific.
(B.2) Social: Other	
Allogroom give, n/s	'**AlloG**'[b]: licking a conspecific.
Allogroom receive, n/s	'**AlloR**'[b]: receipt of licks from a conspecific.
Mutual head rub, n/s	'**MutHdRub**'[b]: mutual head rubbing (head to head) by the focal cow and a conspecific.
All social: give, n	'**SocG**'[c] = 'AlloG' + 'BPG' + 'CG' + 'Chin rest give' (use of chin to exert pressure on the lateral posterior of a conspecific) + 'HBG' + 'HPG' + 'Head rub give' (rub head on a conspecific without reciprocation) + 'HSG' + 'Mount give' (rest chest floor on the back/rump of a conspecific) + 'MutHdButt' + 'MutHdRub'
All social: receive, n	'**SocR**'[c] = 'AlloR' + 'BPR' + 'CR' + 'Chin rest receive' + 'HBR' + 'HPR' + 'Head rub receive' + 'HSR' + 'Mount receive' + 'MutHdButt' + 'MutHdRub'
(C.) Activity	
Transitions, n	'**Trans**'[c]: the total number of changes in behavior a focal cow undergoes during the observation period.
Distance, units	'**Dist**'[c]: the number of units of floor space crossed by the focal cow during the observation period.
(D.) Social Proximity	
Feed barrier: all, s	'FB_All': total time spent at the feed barrier.
Feed barrier: no near neighbour, %	'FB_0NN': time spent at the feed barrier when the focal cow was not in direct contact with any conspecific. '**%FB_0NN**'[a] = ('FB_0NN'/'FB_All') x 100
Feed barrier: two near neighbours, %	'FB_2NN': time spent at the feed barrier when the focal cow was in direct (flank to flank) contact with two conspecifics. '**%FB_2NN**'[a] = ('FB_2NN'/'FB_All') x 100
Lie: no near neighbours, %	'C_0NN': time spent lying while flanked by two unoccupied cubicles. '**%C_0NN**'[a] = ('C_0NN'/'Lie') x 100
Lie: two near neighbours, %	'C_2NN': time spent lying while flanked by two occupied cubicles. '**%C_2NN**'[a] = ('C_2NN'/'Lie') x 100
Feed barrier: open, %	'FB_Open': time spent at the open section of the feed barrier. '**%FB_Open**'[a] = ('FB_O'/'FB_All') x 100

172 [a]inclusion within 24h dataset only; [b]inclusion within 24h and 1hPostM1 datasets; [c]inclusion within 24h, 1hPostM1 and diurnal (hourly) datasets

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

To account for differences in total time visible (i.e. due to variations in time spent within the parlour) data in the '24h' and 'Diurnal' data sets were standardised to either: number per hour visible (behavioral events) or seconds per hour visible (behavioral states). Outliers (± 2SD) were removed prior to data analysis.

2.5. Saliva Collection and SAA

Saliva was collected (Day 3) using a cotton swab (SalivaBio Children's Swab, Item No. 5001.06, Salimetrics) and then immediately stored at -80°C prior to analysis. SAA was measured in saliva from 31 cows, diluted 1:2, using a commercially available kit (Bovine Serum amyloid A protein ELISA Kit, EB0015, Finetest®, Wuhan Fine Biotech Co. Ltd.). To assess the suitability of the kit for use with saliva an assay validation was performed. To determine parallelism (linearity) a displacement curve, produced by double-diluting a pooled saliva sample with assay buffer, was compared to a standard curve. Percentage binding (as a percentage of that recorded for the zero standard) was calculated, in addition to the Log of the standard concentration (SAA standard) and the Log of the inverse of the dilution factor (saliva sample), e.g. 1:4 was transformed to Log(1/4). Parallelism was confirmed using a statistical test for the analysis of covariance (ANCOVA, SPSS). To measure assay accuracy the percentage recovery of exogenous SAA was calculated following the addition of 300 ng/ml SAA standard to a pooled saliva sample. Precision was assessed via intra- and inter-assay coefficients of variation (**CV**); the former was determined following the repeated measurement of aliquots of pooled saliva containing either high (quality control: $\mathbf{QC_{high}}$) or low ($\mathbf{QC_{low}}$) endogenous SAA within the same plate, while the later was determined following the assay of QC_{high} and QC_{low} samples in different plates.

2.6. Statistics

Following tests for normality (Shapiro-Wilk analysis), comparisons between the CTRL and SCM groups were made for all behavioral and physiological measures (Paired samples t-test or Wilcoxon signed-rank tests, IBM SPSS Statistics 24.0). Since the experimental design required the performance of multiple comparisons between measures there was an increased associated risk of Type I errors. Use of Bonferroni correction procedures has been highlighted as problematic (especially for animal behavioral studies, where sample sizes are often small) due to their tendency to increase Type II errors (Nakagawa, 2004). As an alternative to standard correction procedures we, therefore, calculated measures of observed (standardised) effect size in addition to p-values. Effect size measures the strength/magnitude

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

of a relationship and, thereby, helps us to determine the strength of a statistical claim and whether a difference is real (i.e. it enables us to judge biological importance). Hedges' g-value (Equations 1 and 2), also termed 'Cohen's d-value for paired samples' (Hedges, 1981; Cohen, 1988; Nakagawa and Cuthill, 2007) and 95% confidence intervals (**CI**) for effect size (Equations 3 and 4), were calculated for all measures that met the assumptions of normality.

$$g = \frac{\bar{x}_2 - \bar{x}_1}{\sigma_{\text{paired}}} \quad (1)$$

where

$$\sigma_{\text{paired}} = \sqrt{\frac{(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2}{n_1 + n_2 - 2}} \quad (2)$$

$\bar{x}_1$ and $\bar{x}_2$ are the means of the two groups, σ_{paired} is the pooled standard deviation, n is the number of data points, and s^2 is the sample variance.

$$95\%\text{CI} = g - 1.96se_{\text{g}} \text{ to } g + 1.96se_{\text{g}} \quad (3)$$

where

$$se_{\text{g}} = \sqrt{\frac{2(1-r_{1,2})}{n} + \frac{g^2}{2(n-1)}} \quad (4)$$

se_{g} is the asymptotic standard error for the effect size, $n = n_1 = n_2$, and $r_{1,2}$ is the correlation coefficient between the two groups. For all behavioral measures that failed to meet the assumptions of normality, bootstrap effect size values (Hedges' g-value with 95%CI, R = 2000) were computed using the software package 'bootES' (Gerlanc and Kirby, 2012; Kirby and Gerlanc, 2013) and R (Version 3.2.2., www.r-project.org/). Effect size statistics were interpreted as follows: (a) the size of the effect (based upon the estimated g-values: ≤0.39 = small, 0.40 - 0.79 = medium, ≥0.80 = large); (b) statistical significance (attributed to all measures where the associated 95%CI did not contain '0') (Lee, 2016).

Interpretation of statistically non-significant p-values is possible using effect size confidence intervals in combination with the effect size (see Nakagawa and Foster, 2004). To identify measures that failed to reach statistical significance in the current study (24h data set) but that could potentially still be biologically important, we used information from pre-existing literature to set accepted relative difference levels (**RDL%**, Table 2).

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 2: Summary of relative difference levels (RDL%), based upon previous literature, used to ascertain the existence of a biological difference between two groups of cow (CTRL and SCM) for the different behavioral measures

Behavioral Measure	RDL%	Reference
%FB_Open	10	Huzzey et al., 2006
%FB_0NN, %FB_2NN	20	Manson and Appleby, 1990
Feed	10	Dollinger and Kaufmann, 2013
Drink	20	Huzzey et al., 2007
Lie	10	Toaff Rosenstein et al., 2016
Activity: Trans, Dist	10	King et al., 2018; Steensels et al., 2017
Brush	30	Mandel et al., 2017
Comfort	20	Fogsgaard et al., 2012
AlloG	30	Galindo and Broom, 2002
AlloR	80	Galindo and Broom, 2002; Hoonhout et al., 2017
Agonistic social give: all categories	10	Sepulveda-Vares et al., 2016
Agonistic social receive: all categories	10	Neave et al., 2018
Other	20	

For those measures where no relevant literature was available, an RDL% of 20% was employed; this was the average of our other RDL% levels. For each measure, relative difference values (**RDV**) were then calculated using the RDL% and the respective mean value from the CTRL group. $95\%CI_{RDV}$ were calculated using the confidence intervals from the effect size statistics and the (between-group) difference in means. In those cases where the $95\%CI_{RDV}$ did not include the RDV, we conclude (with 95% confidence) that the current study showed no important biological effect for that measure; we refer to these as 'biologically unimportant'. In cases where the $95\%CI_{RDV}$ did include the RDV, we conclude that a difference was inconclusive but plausible; we refer to these as 'biologically inconclusive'. For example, if CTRL cows performed more body pushes than the SCM cows, yet this difference failed to reach statistical significance ($P \geq 0.10$), using p-value alone we would dismiss this behavior as being unaffected by subclinical inflammation. However, if the RDV for this behavior was within the $95\%CI_{RDV}$ range (e.g. RDV = 0.08, $95\%CI_{RDV}$ = -0.08 to 0.27) we can conclude that, although this effect is biologically inconclusive based upon our evidence, the difference may become significant given a larger sample size. Alternatively, if the RDV, in the above example, was 0.3, then we would conclude that the effect was biologically unimportant. To identify correlations between physiological (SCC and SAA) and

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

behavioral measures (24h data set) we performed curve estimation regression statistics (SPSS: ANOVA, coefficient of determination).

3. RESULTS

3.1. Behavioral Differences

3.1.1. Core behavior

Paired t-tests suggested no significant differences in time spent feeding (‘**Feed**’), drinking (‘**Drink**’), or lying (‘**Lie**’) between SCM and CTRL groups (Tables 3 and 4), nor was subclinical mastitis considered to have biologically significant effects on these core maintenance behaviors (‘Feed’: RDV = 82.39 s, $95\%CI_{RDV}$ = -0.03 to 0.03 s; ‘Drink’: RDV = 6.86 s, $95\%CI_{RDV}$ = -1.33 to 3.99 s; ‘Lie’: RDV = 200.19 s, $95\%CI_{RDV}$ = -23.54 to 79.67 s). Over the 24h period SCM cows spent more time lying with their head on their flank than CTRL cows (‘**HOF**’, Paired t-test: p = 0.046; Hedges’ g: significant medium effect). CTRL cows were more active, performing more behavioral transitions (‘**Trans**’ 24h, p = 0.040; large; 1hPostM1, p = 0.041; large) and moving over a greater distance than the SCM cows (‘**Dist**’: 24h, p = 0.032, large; 1hPostM1, p = 0.098; medium).

Table 3: Measures of core behavior in two groups of cow (CTRL: n = 17; SCM: n = 17) over 24h. All units transformed to ‘per hour visible’

Measure	CTRL		SCM		Effect size statistics		
	Mean	SD	Mean	SD	Hedges’ g	95%CI	Size of effect
Lie[1], s	2001.93[a]	211.62	2092.46[a]	367.64	-0.31	-0.26 - 0.88	Small, ns
HOF[1], s	143.48[a]	49.74	178.67[b]	74.84	-0.57	0.06 - 1.09	Med, sig
Drink[1], s	34.32[a]	12.16	30.16[a]	14.64	0.32	-0.32 - 0.96	Small, ns
Feed[1], s	823.87[a]	167.46	823.80[a]	281.18	0.00	-0.46 - 0.46	Small, ns
Trans[1], n	55.59[a]	8.24	48.55[b]	7.80	0.91	0.08 - 1.74	Large, sig
Dist[1], unit	8.20[a]	1.88	6.75[b]	1.49	0.88	0.11 - 1.65	Large, sig
Comfort[1], s	21.16[a]	7.94	26.00[a]	10.77	-0.27	-0.58 - 1.12	Small, ns
ExpEnv[1], s	55.38[a]	20.87	49.96[a]	14.45	0.31	-0.24 - 0.86	Small, ns
Brush[2], s	28.99[a]	43.87	11.85[a]	9.01	0.53	-0.16 - 0.96	Med, ns

[a-b]Mean values in the same row with different superscripts differ (P <0.05)

[1]Statistical analysis performed using Paired t-test

[2]Statistical analysis performed using Wilcoxon SR test

Although SCM cows tended only to perform statistically more ‘Comfort’ behavior following morning milking (1hPostM1: p = 0.073, medium), confidence intervals for effect size

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

differences classified both measures of self-grooming to be biologically inconclusive over 24h ('Brush': RDV = 8.70 s, $95\%CI_{RDV}$ = -2.74 to 16.45 s; 'Comfort': RDV = 4.23 s, $95\%CI_{RDV}$ = -2.81 to 5.42 s). This indicates that, given a larger sample size, significant differences may have become apparent (i.e. higher brush use by CTRL cows and more comfort behavior by SCM cows). No difference in environmental exploration was evident between the groups over the 24h period, nor was this deemed to be biologically important in the context of this study ('**ExpEnv**': RDV = 11.08 s, $95\%CI_{RDV}$ = -1.30 to 4.66 s); however, CTRL cows did explore their environment more than SCM cows 1hPostM1 ('ExpEnv': p = 0.031, large).

Table 4: Measures of maintenance behavior in two groups of cow (CTRL: n = 17; SCM: n = 17) during 60 mins following morning milking (1hPostM1)

Measure	Control		SCM		Effect size statistics		
	Mean	SD	Mean	SD	Hedges' g	95%CI	Size of effect
Dist[1], unit	15.87[a]	6.57	12.53[b]	4.03	-0.63	-0.08 - 1.34	Med, ns
Comfort[1], s	29.20[a]	25.61	47.40[b]	44.15	0.52	0.01 - 1.03	Med, sig
ExpEnv[1], s	71.81[a]	44.15	39.31[c]	27.79	-0.91	0.11 - 1.71	Large, sig
Trans[2], n	99.13[a]	34.79	71.67[c]	24.58	-0.89	0.21 - 1.62	Large, sig

[a-c]Mean values in the same row with different superscripts differ ([b]P<0.10; [c]P <0.05)

[1]Statistical analysis performed using Paired t-test

[2]Statistical analysis performed using Wilcoxon SR test

3.1.2. Social behavior and pen-mate proximity

No differences were observed in the overall performance (24h), or receipt, of social behavior between groups (Tables 5 and 6). Overall performance of cumulative social behavior was classified as biologically unimportant in the context of this dataset, while receipt of social behavior was biologically inconclusive ('**SocG**': RDV = 0.50, $95\%CI_{RDV}$ = -0.26 to 0.43; '**SocR**': RDV = 0.44, $95\%CI_{RDV}$ = -0.21 to 0.64). CTRL cows performed more social exploration ('**ExpSoc**': 24h, p = 0.009, large; 1hPostM1, p = 0.026, medium), more head butts ('**HBG**': 24h, p = 0.043; 1hPostM1, p = 0.055, medium) and more head pushes ('**HPG**': 1hPostM1, p = 0.027, large) than the SCM cows. Confidence intervals classified all other agonistic measures which failed to reach statistical significance as being biologically inconclusive ('FocSocG': RDV = 0.40, $95\%CI_{RDV}$ = -0.31 to 0.73; 'BPG': RDV = 0.08, $95\%CI_{RDV}$ = -0.08 to 0.27; 'CG': RDV = 0.02, $95\%CI_{RDV}$ = 0.00 to 0.15; 'HPG': RDV = 0.03, $95\%CI_{RDV}$ = -0.03 to 0.18; 'HSG': RDV = 0.17, $95\%CI_{RDV}$ = -0.12 to 0.63).

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 5: Measures of social behavior recorded from two groups of cow (CTRL: n = 17; SCM: n = 17) over 24h. All units (except %) transformed to 'per hour visible'.

Measure	CTRL		SCM		Effect Size Statistics		
	Mean	***SD***	**Mean**	***SD***	**Hedges' g**	**95%CI**	**Size of effect**
ExpSoc[1], s	18.66[a]	*12.26*	9.14[c]	*5.40*	1.04	0.30 – 1.78	Large, sig
BPR[1], n	0.83[a]	*0.29*	0.79[a]	*0.38*	0.11	-0.31 – 0.53	Small, ns
CG[1], n	0.23[a]	*0.24*	0.11[b]	*0.12*	0.62	-0.01 - 1.25	Med, ns
CR[1], n	0.32[a]	*0.25*	0.17[c]	*0.10*	0.84	0.02 - 1.66	Large, sig
HBG[1], n	1.07[a]	*0.87*	0.64[c]	*0.41*	0.65	0.10 - 1.20	Med, sig
HBR[1], n	0.70[a]	*0.38*	0.59[a]	*0.26*	0.37	-0.45 – 1.19	Small, ns
%HBR+D[1], %	69.85[a]	*15.51*	52.66[c]	*16.78*	1.10	0.46 - 1.74	Large, sig
HPR[1], n	0.23[a]	*0.20*	0.12[b]	*0.15*	0.64	-0.01 – 1.29	Med, ns
HSG[1], n	1.73[a]	*0.84*	2.26[a]	*1.41*	-0.47	-0.23 – 1.18	Med, ns
HSR[1], n	1.39[a]	*0.90*	2.01[b]	*1.50*	-0.51	0.01 – 1.02	Med, sig
%HSR+D[1], %	32.88[a]	*13.32*	24.71[c]	*13.59*	0.63	0.03 - 1.22	Med, sig
FocSocG[1], n	3.98[a]	*2.59*	3.33[a]	*1.47*	0.32	-0.48 - 1.12	Small, ns
FocSocR[1], n	3.41[a]	*1.17*	3.85[a]	*1.84*	-0.30	-0.30 – 0.90	Small, ns
AlloG[1], s	18.17[a]	*13.97*	10.09[c]	*9.65*	0.70	0.05 – 1.35	Med, sig
SocR[1], n	4.40[a]	*1.06*	5.03[a]	*2.49*	-0.34	-0.34 – 1.02	Small, ns
%FB_0NN[1], %	41.41[a]	*12.91*	36.01[b]	*13.02*	0.43	-0.02 – 0.88	Med, ns
%FB_2NN[1], %	24.59[a]	*9.72*	30.02[c]	*9.77*	-0.56	0.11- 1.01	Med, sig
%FB_Open[1], %	82.22[a]	*11.97*	90.14[c]	*7.66*	-0.81	0.10 - 1.53	Large, sig
%C_0NN[1], %	38.75[a]	*20.29*	35.82[a]	*20.66*	0.43	-0.02 – 0.88	Med, ns
BPG[2], n	0.83[a]	*0.82*	0.53[a]	*0.32*	0.47	-0.25 – 0.90	Med, ns
%BPR+D[2], %	51.54[a]	*14.04*	53.68[a]	*17.90*	-0.11	-0.53 – 0.85	Small, ns
%CR+D[2], %	84.44[a]	*13.39*	76.85[a]	*23.25*	0.39	-0.32 – 1.10	Small, ns
HPG[2], n	0.30[a]	*0.41*	0.13[a]	*0.13*	0.52	-0.15 - 1.04	Med, ns
%HPR+D[2], %	52.79[a]	*25.53*	22.31[c]	*35.00*	0.96	-0.02 – 2.23	Large, ns
%FocSocR+D[2], %	49.10[a]	*11.07*	39.08[c]	*11.56*	0.87	0.11 – 1.63	Large, sig
MutHdButt[2], s	11.32[a]	*11.93*	5.33[a]	*3.99*	0.66	-0.03 - 1.34	Med, ns
AlloR[2], s	13.77[a]	*11.36*	15.88[a]	*14.70*	-0.16	-0.54 – 0.79	Small, ns
SocG[2], n	5.02[a]	*2.50*	4.53[a]	*2.22*	0.20	-0.53 – 0.87	Small, ns
%C_2NN[2], %	21.90[a]	*19.63*	18.58[a]	*20.56*	0.08	-0.81 - 0.58	Small, ns

[a-c]Mean values in the same row with different superscripts differ ([b]P<0.10; [c]P <0.05)

[1]Statistical analysis performed using Paired t-test

[2]Statistical analysis performed using Wilcoxon SR test

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 6: Measures of social behavior observed between two groups of cow (CTRL: n = 17; SCM: n = 17) during 60 mins following morning milking (1hPostM1)

Measure	Control		SCM		Effect Size Statistics		
	Mean	*SD*	Mean	*SD*	Hedges' g	95%CI	Size of effect
FocSocG[1], n	10.69[a]	*9.02*	5.81[b]	*4.68*	-0.70	-0.05 – 1.45	Med, ns
FocSocR[1], n	4.67[a]	*2.64*	2.47[c]	*1.92*	-0.99	0.04 – 1.93	Large, sig
ExpSoc[2], s	21.19[a]	*28.75*	6.50[c]	*7.63*	-0.68	0.06 – 1.07	Med, sig
BPR[2], n	3.25[a]	*2.52*	1.19[c]	*1.47*	-0.98	0.29 – 1.67	Large, sig
HBG[2], n	1.69[a]	*2.44*	0.50[b]	*0.63*	-0.65	0.10 – 1.09	Med, sig
HBR[2], n	1.13[a]	*1.41*	0.31[b]	*0.60*	-0.73	0.10 – 1.40	Med, sig
HPG[2], n	0.94[a]	*1.29*	0.13[c]	*0.34*	-0.84	0.24 – 1.38	Large, sig
AlloR[2], s	37.56[a]	*66.11*	1.88[c]	*3.18*	-0.74	0.44 – 1.11	Med, sig
SocR[2], n	12.13[a]	*7.43*	6.60[c]	*3.92*	-0.91	0.17 – 1.51	Large, sig

[a-c]Mean values in the same row with different superscripts differ ([b]P<0.10; [c]P <0.05)

[1]Statistical analysis performed using Paired t-test

[2]Statistical analysis performed using Wilcoxon SR test

In the hour after first milking (1hPostM1) CTRL cows received significantly more social behavior ('SocR': p = 0.038, large), agonistic behavior ('**FocSocR**': p = 0.049, large), body pushes ('**BPR**': p = 0.022, large) and head butts ('**HBR**': p = 0.080, medium) than SCM cows. Overall (24h), CTRL cows also received more challenges ('**CR**': p = 0.050, large), while SCM cows received more head swipes ('**HSR**': p = 0.070, medium). With the exception of 'BPR', which was classified as biologically unimportant ('BPR': RDV = 0.08, $95\%CI_{RDV}$ = -0.01 - 0.02), all other measures that failed to reach statistical significance were deemed to be biologically inconclusive ('HBR': RDV = 0.07, $95\%CI_{RDV}$ = -0.05 to 0.13; 'HPR': RDV = 0.02, $95\%CI_{RDV}$ = -0.00 to 0.14; 'FocSocR': RDV = 0.34, $95\%CI_{RDV}$ = -0.13 to 0.40; 'MutHdButt': RDV = 1.13 s, $95\%CI_{RDV}$ = -0.19 to 8.03 s).

CTRL cows were more reactive, i.e. more likely to be displaced, than SCM cows following the receipt of agonistic behavior cumulatively ('**%FocSocR+D**': p = 0.010, large), a head butt ('**%HBR+D**': p <0.001, large), head push ('**%HPR+D**': p = 0.037, large), or a head swipe ('**%HSR+D**': p = 0.048, medium). All reactivity measures that failed to reach statistical significance were classified as biologically unimportant in the context of this study ('%BPR+D': RDV = 5.15%, $95\%CI_{RDV}$ = -1.13 to 1.82%; '%CR+D': RDV = 8.44%, $95\%CI_{RDV}$ = -2.43 to 8.35%). Although CTRL cows were observed to allogroom significantly more than SCM cows during the 24h period ('**AlloG**': p = 0.047, medium), no

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

overall difference in the receipt of allogrooming was evident, and this measure was classified as biologically unimportant (‘**AlloR**’: RDV = 11.02 s, 95%CI_{RDV} = -1.14 to 1.67 s). CTRL cows were allogroomed more than SCM cows 1hPostM1 ($p = 0.012$, medium).

SCM cows spent a significantly greater proportion of their time at the feed passage flanked by two neighbours (‘**%FB_2NN**’: $p = 0.019$, medium), and a significantly greater proportion of their time at the open section of the feed barrier (‘**%FB_Open**’: $p = 0.032$, large), than did the CTRL cows. All other measures of social proximity were classified as biologically unimportant within the context of this study (‘%FB_0NN’: RDV = 8.28%, 95%CI_{RDV} = -0.11 to 4.75%; ‘%C_0NN’: RDV = 7.75%, 95%CI_{RDV} = -0.06 to 2.58%; ‘%C_2NN’: RDV: 4.38%, 95%CI_{RDV} = -2.69 to 1.93%).

3.2. Differences in Diurnal Behavior Patterns

A combination of paired-sample and post-hoc (effect size statistic) testing between the two groups revealed differences in diurnal patterns of activity and social behavior (Figure 2). The SCM cows were more active between: (a) 00:00 and 01:00h, when they performed more exploratory behavior and moved over a greater distance than the CTRL cows, and (b) 13:00 and 14:00h, when they performed more exploratory and social behavior. CTRL cows were more active than the SCM cows during three periods: (a) between 02:00 and 03:00h they performed more social exploration, received more social behavior, and walked a greater distance, (b) during 05:00 to 06:00h they lay less, moved further, and performed more behavioral transitions, exploratory, and agonistic behavior, and (c) between 16:00 and 17:00h they performed more behavioral transitions and performed/received more agonistic behavior.

INSERT FIGURE 2 NEAR HERE

3.3. Correlations Between Physiology and Behavior

3.3.1. Assay validation

Parallelism ($F_{1,9} = 3.46$, $p > 0.05$) was confirmed between serial dilutions of saliva (range: 1:4 to 1:64) and SAA standards (range: 0, 9.38, 18.75, 37.5, 75, 150, 300 ng/ml), indicating that the ELISA kit was suitable for use with bovine saliva. Recovery of 300 ng/ml SAA from a spiked saliva sample was 93.76 ± 4.63% (n = 10). The intra-assay CV was 3.09% (250.87 ± 7.75 ng/ml, n = 10) for QC_{low} and 4.68% (1360.33 ± 63.70 ng/ml, n = 10) for QC_{high}. The

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

inter-assay CV was 2.77% (246.06 ± 6.81 ng/ml, n = 2) for QC_{low} and 3.89% (1323.96 ± 51.43 ng/ml, n = 2) for QC_{high}.

3.3.2. SAA and SCC

The average SCC per group was: CTRL = 48.29 ± 28.33 (x1000 cells/ml); SCM = 351.12 ± 176.73 (x1000 cells/ml). A trend was evident for a higher concentration of salivary SAA in the SCM cows (CTRL = 343.42 ± 269.60 ng/ml, SCM = 519.59 ± 315.43 ng/ml; $t_{1,12}$ = 1.93, p = 0.076), and a weak positive logarithmic relationship was evident between SCC and SAA ($F_{(1,26)}$ = 6.26, p = 0.019; R^2 = 0.194; y = 113.99ln(x) - 81.384, Figure 3).

INSERT FIGURE 3 NEAR HERE

3.3.3. SAA, SCC and behavior

Of the 34 behavioral measures to have had correlation analyses calculated against SAA and SCC, 24 were significant; most relationships identified were weak (Table 7). No correlation (SAA or SCC) was evident for: 'HOF', 'BPG', 'CG', 'HBR', 'HPG', '%BPR+D', 'AlloR', '%FB_2NN', 'Brush' or 'Comfort'. A weak positive correlation between SAA and 'Lie', and moderate negative correlations between SAA and both 'Feed' and 'Drink', indicate that as systemic inflammation rose consumption dropped and lying increased. Positive correlations between SAA and both 'ExpEnv' and 'ExpSoc', suggest that cows with higher inflammation levels were more explorative. However, a negative correlation between SCC and 'ExpSoc' was also observed. Quadratic relationships were evident between SCC and both 'Trans' and 'Dist'; these described an initial drop in activity, as SCC increased to approximately 300 (x1000 cells/ml), followed by an increase as SCC continued to rise (Figure 4). Positive correlations between SAA and 'SocR', 'FocSocR', 'CR', and 'HPR', indicate that cows with higher levels of systemic inflammation were receiving more socially agonistic behaviors.

INSERT FIGURE 4 NEAR HERE

Negative correlations between SCC and both 'BPR' and 'HPR' imply that certain agonistic behaviors were primarily directed at cows without intra-mammary inflammation. Negative correlations between SAA and 'SocG', 'FocSocG' and 'HSG' indicate that the performance of social behavior decreased with systemic inflammation. Increasing SCC levels were also associated with the performance of fewer head butts and the receipt of more head swipes.

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 7: Significant correlations between behavioral measures (24h) and two markers of inflammation and infection, one from saliva (SAA) and one from milk (SCC): curve estimation regression statistics (ANOVA, coefficient of determination) and the equation for the relationship (based upon the line of best fit).

Behavior	Correlation with Inflammatory Marker
Feed, s	SAA: $F_{(1,28)} = 16.05$, p <0.001; $R^2 = 0.364$; $y = 1048.6e^{-7E-04x}$
Drink, s	SAA: $F_{(1,26)} = 21.83$, p <0.001; $R^2 = 0.456$; y = -7.077ln(x) + 71.135
Lie, s	SAA: $F_{(1,28)} = 9.69$, p = 0.004; $R^2 = 0.257$; y = 0.563x + 1830
Trans, n	SCC: $F_{(2,29)} = 6.21$, p = 0.006; $R^2 = 0.300$; $y = 0.0002x^2 - 0.101x + 59.461$
Dist, units	SCC: $F_{(2,30)} = 4.11$, p = 0.026; $R^2 = 0.215$; $y = 4E-05x^2 - 0.021x + 9.146$
ExpEnv, s	SAA: $F_{(1,28)} = 5.78$, p = 0.023; $R^2 = 0.171$; y = 8.690ln(x) + 7.484
ExpSoc, s	SAA: $F_{(1,28)} = 5.57$, p = 0.025; $R^2 = 0.166$; y = 0.021x + 6.815 SCC: $F_{(1,29)} = 10.17$, p = 0.003; $R^2 = 0.260$; $y = 16.99e^{-0.003x}$
BPR, n	SCC: $F_{(1,30)} = 4.03$, p = 0.054; $R^2 = 0.119$; $y = 0.8818e^{-0.001x}$
CR, n	SAA: $F_{(1,29)} = 3.93$, p = 0.057; $R^2 = 0.119$; y = 0.0003x + 0.165
HBG, n	SCC: $F_{(1,30)} = 5.50$, p = 0.026; $R^2 = 0.155$; $y = 0.8708e^{-0.002x}$
HPR, n	SAA: $F_{(1,27)} = 4.86$, p = 0.036; $R^2 = 0.153$; y = 0.0002x + 0.062 SCC: $F_{(1,31)} = 5.87$, p = 0.021; $R^2 = 0.159$; y = -1.990x + 5.520
HSG, n	SAA: $F_{(1,27)} = 3.79$, p = 0.062; $R^2 = 0.123$; $y = 2.2425e^{-8E-04x}$
HSR, n	SAA: $F_{(1,28)} = 3.48$, p = 0.072; $R^2 = 0.111$; y = -0.389ln(x) + 3.858 SCC: $F_{(1,30)} = 7.74$, p = 0.023; $R^2 = 0.161$; y = 0.0002x + 1.18
FocSocR, n	SAA: $F_{(1,26)} = 3.68$, p = 0.066; $R^2 = 0.124$; y = 0.588ln(x) - 0.140
FocSocG, n	SAA: $F_{(1,27)} = 3.27$, p = 0.068; $R^2 = 0.118$; $y = 4.097e^{-6E-04x}$
%CR+D, %	SAA: $F_{(1,29)} = 3.62$, p = 0.068; $R^2 = 0.122$; $y = 91.165e^{-4E-04x}$
%HBR+D, %	SAA: $F_{(1,29)} = 5.64$, p = 0.024; $R^2 = 0.163$; $y = 72.616e^{-5E-04x}$ SCC: $F_{(1,31)} = 8.81$, p = 0.006; $R^2 = 0.221$; y = -7.36ln(x) + 95.594
%HPR+D[1], %	SAA: $F_{(1,22)} = 3.68$, p = 0.068; $R^2 = 0.143$; y = 0.002x + 0.422
%HSR+D, %	SAA: $F_{(1,29)} = 3.29$, p = 0.080; $R^2 = 0.102$; y = -0.015x + 34.655
%FocSocR+D, %	SAA: $F_{(1,27)} = 4.93$, p = 0.035; $R^2 = 0.154$; y = -0.016x + 50.817 SCC: $F_{(1,30)} = 8.54$, p = 0.007; $R^2 = 0.222$; y = -4.907ln(x) + 67.391
AlloG, s	SCC: $F_{(1,29)} = 3.50$, p = 0.072; $R^2 = 0.108$; y = -0.023x + 17.91
SocG, n	SAA: $F_{(1,28)} = 6.13$, p = 0.020; $R^2 = 0.180$; $y = 6.0245e^{-7E-04x}$
SocR, n	SAA: $F_{(1,26)} = 5.75$, p = 0.024; $R^2 = 0.187$; y = 0.003x + 3.222
%FB_Open, %	SAA: $F_{(1,28)} = 4.08$, p = 0.053; $R^2 = 0.127$; y = 3.244ln(x) + 67.752 SCC: $F_{(1,31)} = 7.71$, p = 0.009; $R^2 = 0.199$; y = 0.024x + 80.896

[1]Log_{10} transformation prior to regression analysis

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Negative correlations were observed between SAA and '%FocSocR+D', '%CR+D', '%HBR+D' and '%HSR+D', and between SCC and both '%FocSocR+D' and '%HBR+D'. This indicates that cows with greater inflammation were less likely to move away (i.e. be displaced) after receiving agonistic behavior, suggesting lower social reactivity. However, a positive correlation between SAA and '%HPR+D' suggests that, following receipt of a head push, cows with greater systemic inflammation were displaced more frequently. A negative correlation between SCC and 'AlloG' indicates that the performance of allogrooming decreased with increased mammary inflammation. Finally, positive correlations between both inflammatory markers and '%FB_Open' reveal that the self-locking feed barriers were used less as inflammation increased.

4. DISCUSSION

The main purpose of this study was to identify salivary SAA, social and other behavioral changes associated with subclinical inflammation (mastitis) in cows. Salivary SAA was shown to have potential as a marker of low-level systemic inflammation because levels were found to be higher in cows with subclinical mastitis, and higher levels were associated with several key sickness behaviors. Furthermore, SCM cows displayed lower 'activity', 'sociality' (including the performance and receipt of multiple social behaviors) and 'social reactivity', and demonstrated a shift in activity peaks for several behaviors to quieter times of the day.

4.1. Salivary SAA

Positive correlations between SCC and non-salivary SAA have often been reported from cows with clinical and sub-clinical mastitis (serum: des Roches et al., 2017; milk: O'Mahony et al., 2006; Akerstedt et al., 2007; Pyörälä et al., 2011) and we report here, for the first time, the same for salivary SAA. SAA in saliva thus appears to offer potential as a non-invasive means of detecting subclinical infection. During field conditions, several bacterial strains can cause mastitis of varying duration and degree (Verbeke et al., 2014), and it is likely that the concentration of SAA in saliva will vary accordingly. Pyörälä et al. (2011) detected significant differences in SAA (milk) collected from cows with spontaneous mastitis caused by different pathogens; low SAA was associated with *A. pyogenes*, while high concentrations were associated with *E. coli*.

4.2. Social Behavior

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Although no differences in cumulative social behavior given or received were evident between the two groups overall (24h), the CTRL cows received significantly more social behavior than the SCM cows following morning milking (1hPostM1); this provides evidence for diurnal differences in behavior. By dividing social behavior into the broad categories of socio-negative (i.e. agonistic competitive) and socio-positive (affiliative) we were able to identify specific disparities.

4.2.1. Agonistic competitive behavior

Sick cows are often reported to perform fewer agonistic interactions and competitive displacements from the feed-bunk (bacterial lameness: Galindo and Broom, 2000; 2002; sub-clinical metritis: Huzzey et al., 2007; Patbandba et al., 2012; clinical and sub-clinical mastitis: Sepúlveda-Varas et al., 2014; 2016), and from cubicles (Jensen and Proudfoot, 2017), than healthy individuals. In addition, sick cows often receive more agonism, and are displaced more frequently, than healthy cows (bacterial lameness: Galindo and Broom, 2002; metritis: Patbandba et al., 2012; Neave et al., 2018; Lomb et al., 2018; metritis and sub-clinical ketosis: Schirmann et al., 2016). On this basis we predicted our SCM cows to also perform less and receive more agonistic behavior than the CTRL cows. Counter to these expectations the CTRL group received more challenges (24h), and more head butts, body pushes and total aggression (1hPostM1), than the SCM group, supported by negative correlations between SCC and the receipt of both head and body pushes. Presumably, the healthy animals partook in more physical contact and jostling as part of actively re-establishing dominance, since aggressive competitive interactions are key to establishing and maintaining social order within dynamic groups (Val-Laillet et al., 2008).

The receipt of head swipes was the one agonistic measure that was significantly higher in our SCM group (24h). Since this is a common social behavior, occurring almost exclusively at the feed barrier as part of feed competition (i.e. a means of displacing immediate neighbours), this finding does correspond with predictions of ‘sickness’ and the wider literature. Interestingly, we also observed positive correlations between SAA and several measures of social and agonistic receipt (‘SocR’, ‘FocSocR’, ‘CR’, ‘HPR’), indicating that cows with higher levels of systemic inflammation also were more frequently the recipients of agonistic behavior. We cannot discount the possibility that SAA upregulation occurred within our CTRL group due to early undiagnosed non-mastitic infection or following exposure to social stress. Upregulation of C-Reactive Protein (an APP known to increase during illness and

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

stress) has been reported in zoo-housed gorillas following an aggressive encounter (Fuller and Allard, 2018). In the current study saliva samples were collected the day after behavioral observations were made, therefore an elevation in SAA may also occur as a consequence of agonistic encounters experienced during the previous day.

In line with the hypothesis that social behavior should decrease with inflammation/sickness, and our observation that CTRL cows performed more agonistic behavior, SAA was negatively correlated with ‘SocG’, ‘FocSocG’ and ‘HSG’, and SCC was negatively correlated to ‘HBG’. Although social rankings were not calculated in the current study it is possible that the social rank of an individual could be influenced by the effects of disease due to a loss of competitive vigour. Dominant animals frequently displace subordinate cows from the feed barrier (DeVries et al., 2004; Huzzey et al., 2006), and subordinate animals adjust their eating patterns accordingly (DeVries et al., 2004). In our study CTRL cows performed more agonistic behavior immediately before the first milking (05:00h) and mid-afternoon (16:00h), while SCM cows performed more prior to the second milking (13:00h). Focusing activities outside of peak times may be a means of avoiding agonistic interactions with socially dominant individuals but, due to high stocking densities, there will always be an immediate social environment to manage. It is conceivable, but beyond the reach of our data, that agonistic behavior performed by the SCM cows was aimed at other sick or low-ranking individuals employing similar competitive tactics.

4.2.2. Social reactivity

Llonch et al. (2018) identified a group of cows that were more reactive to the presence of conspecifics at the feed barrier; frequent feeding interruptions lead to shorter, but more frequent, visits to the feeder. Since subordinate or sick cows are more likely to engage in avoidance behavior in response to social confrontation (Huzzey et al., 2006; Goldhawk et al., 2009; Proudfoot et al., 2009) we may anticipate such individuals to also display greater reactivity (i.e. be more likely to move away when challenged at the feeder). However, in the current study the opposite was true; CTRL cows were more likely to be displaced than SCM cows following the receipt of agonistic behavior. Not only were CTRL cows more reactive than SCM cows, but reactivity appeared to decrease with increasing inflammation (negative correlations were observed between SAA and ‘%FocSocR+D’, ‘%CR+D’, ‘%HBR+D’ and ‘%HSR+D’, and between SCC and both ‘%FocSocR+D’ and ‘%HBR+D’). This observation could be explained by social environment. Choice of feeding position has been shown to be

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

affected by dominance relationships; dissimilar neighbours (low/high rank) are known to maintain a greater distance of separation than individuals of similar rank (Manson and Appleby, 1990). If the CTRL cows were less discriminatory, regarding their social environment, then they may have been more likely to receive aggressive encounters from dominant individuals and reacted accordingly. Conversely, if the SCM cows proactively avoided dominants, and preferentially selected the company of other sick or lower ranking cows, then moderate competitive aggression received from an individual of similar standing may have been tolerable (i.e. not elicited displacement).

4.2.3. Affiliative behavior

Very little research has been conducted on allogrooming and illness in cows. Galindo and Broom (2002) observed lame cows to be allogroomed more than non-lame cows, and this was interpreted as a self-instigated coping strategy triggered by pain/discomfort. Cows appear to find comfort in being licked; individuals who solicit more licking are licked more frequently (Benham, 1984). We predicted that allogrooming would be lower in the SCM group since subordinate individuals are licked, and lick, less frequently than high ranking individuals (Napolitano et al., 2007), allogrooming decreases more in low-ranking individuals under conditions of increased competition (Val-Laillet et al., 2008), and mild 'sickness' is likely to reduce motivation for luxury behavior. As hypothesised, CTRL cows performed more allogrooming than SCM cows overall (24h), confirmed by a negative correlation between SCC and 'AlloG'; in addition, they were also allogroomed more than SCM cows in the hour following morning milking. Since social grooming serves a variety of functions in cattle, including roles in hygiene, the provision of pleasure, the maintenance of social bonds and in lowering social tension (Sato et al., 1991; 1993), it is possible that prolonged suppression of this behavior could have negative implications for welfare and fitness.

4.2.4. Social avoidance

Sickness-driven social avoidance is well documented in lab-animals and humans, and can be predicted by the action of pro-inflammatory cytokines on the CNS (Kent et al., 1992; Bluthe et al., 1996; Dantzer and Kelley, 2007; Arakawa et al., 2010). Due to the limited opportunity for social avoidance within intensive systems this behavior has been relatively understudied in sick farm animals. In the current study CTRL cows performed more social exploration than the SCM cows (and a negative correlation was evident between SCC and 'ExpSoc'),

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

potentially due to the SCM cows actively avoiding social interaction. The unexpected weak positive correlation between SAA and 'ExpSoc' may be, at least partially, explained by the presence of both pre-clinical and post-clinical cows within our focal group; i.e. early-stage mastitic cows (low SAA), demonstrating sickness-driven reductions in 'ExpSoc', in combination with individuals in remission (high SAA), demonstrating normal baseline 'ExpSoc' (see Section 4.4.4).

The prevalence of agonistic behavior at the feed barrier is known to be influenced by barrier design; self-locking yokes have vertical bars which separate the necks of adjacent cows, and these are better at reducing competitive interactions (displacements) compared to open post-and-rail barriers (Endres et al., 2005; Huzzey et al., 2006). In the current study SCM cows spent a significantly greater proportion of their time feeding at the open section of the barrier than did the CTRL cows, and a positive correlation between SAA and '%FB_Open' indicates this preference to increase with rising systemic inflammation. Although this appears to be counter-intuitive, other factors are likely to contribute to the choice of feeding location; e.g., in the open section cows have better visibility and are more quickly able to withdraw from potential agonistic interactions.

4.3. Activity

The observation that our SCM cows made fewer behavioral transitions and moved over a shorter distance than CTRL cows is in agreement with other studies that describe reduced activity prior to the clinical diagnosis of mastitis (Fogsgaard et al., 2012; Kester et al., 2015; Stangaferro et al., 2016; Veissier et al., 2017; King et al., 2018). The quadratic relationships between SCC and both 'Trans' and 'Dist' described in the current study are of interest because mastitic cows have also been reported to display increased activity (Siivonen et al., 2011; Medrano-Galarza et al., 2012), presumably due to udder discomfort and an associated reduction in lying time. Jadhav et al. (2018) argue that the threshold SCC value to delineate subclinical mastitis from normal should be 310, rather than 200 (x1000 cells/ml), as conventionally judged (e.g. Madouasse et al., 2010). This higher value closely corresponds with the parabola vertex in both quadratic plots (Figure 4), of approx. 300 (x1000 cells/ml); i.e. the point at which activity once again begins to rise.

The circadian rhythm of cow activity is known to become disrupted during disease (Veissier et al., 1989; 2017; Kauppi, 2014). Veissier et al. (2017) observed that diseased cows may not

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

consistently decrease their activity, but instead focus their activities within specific time periods; mastitic cows were observed to be hyperactive throughout the day, whereas lame cows were hyperactive at night. We identified two periods during which our SCM cows were more active than the CTRL animals (00:00 to 01:00h and 13:00 to 14:00h); presumably these represented quieter periods, when a proportion of the herd, including socially dominant individuals, were resting.

4.4. Core and Non-Social Behavior

4.4.1. Ingestion

Changes in feeding behavior have long been used to diagnose the onset of illness (Weary et al., 2009). Although we observed a negative correlation between SAA and feeding duration, as would be hypothesised to occur with sickness, the average inflammatory response within our SCM group overall was not sufficiently pronounced to trigger obvious anorexia, as compared to healthy controls. Gonzalez et al. (2008) report variability in feeding behavior relating to naturally occurring udder disorders; some cows demonstrated a decrease in feeding duration with the onset of mastitis, while others showed no change. It is possible that aspects of feeding behavior, other than duration, may have been altered. Barn-housed cattle demonstrate highly synchronised feeding activity, with large peaks in both feeding and social competition coinciding with fresh food delivery, and smaller peaks following milking (DeVries and von Keyserlingk, 2005; Dollinger and Kaufmann, 2013). Mastitic cows, presumably to avoid adverse social interactions, have been shown to feed at less popular times such as early afternoon (Schirmann et al., 2016). Our study identified such a period, between 13:00 and 14:00h (immediately prior to second milking) as one in which the SCM cows fed for longer than the CTRL cows. Sepúlveda-Varas et al. (2016) observed a decrease in feed intake (but not duration) prior to the diagnosis of clinical mastitis which may be attributed to underlying malaise.

Water and feed intake are positively related in cattle (Kume et al., 2010); however, drinking tends to be less affected by health than feeding (Hart, 1988). Water is more immediately vital for maintaining bodily functions (Kyriazakis and Tolkamp, 2011), and since drinking takes less time than food consumption it is less at risk from social competition at the trough (Huzzey et al., 2007). Although a reduction in water consumption has been reported in cows with mastitis (Lukas et al., 2008; Siivonen et al., 2011), and we observed a negative correlation between SAA and drinking duration, the level of systemic inflammation within

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

our SCM group may have been too low, and/or our sample size too small, to induce a group difference.

4.4.2. Lying

Lying is a highly prioritised behavior in cattle due to its importance in rumination (Jensen et al., 2004; 2005; Munksgaard et al., 2005); dairy cows spend approximately 11h/day recumbent (Ito et al., 2009; 2010). Increased lying duration, as a means of conserving energy and facilitating recovery, is a key adaption for sickness, and a positive correlation between SAA and 'Lie' was evident within our test population. Although extended lying duration has been frequently reported during cattle illness (e.g. BRD: Toaff Rosenstein et al., 2016; moderate lameness: Weigele et al., 2017; metritis: Huzzey et al., 2007; Sepúlveda-Varas et al., 2014; Barragan et al., 2018), lying may decrease during mastitis (Yeiser et al., 2012; Medrano-Galarza et al. 2012; Fogsgaard et al., 2012; 2015), presumably due to udder pain (Cyples et al., 2012). Although we observed no difference in 'Lie' between our two groups, the SCM cows were observed to lie with their heads held against their flank more than the CTRL cows. This posture is primarily associated with rapid eye movement (REM) sleep; however, cows are also known to display non-rapid eye movement (NREM) sleep and drowsing in this position (Ternman et al., 2013), and NREM (deep) sleep often increases during infection (Bryant et al., 2004; Opp, 2005).

4.4.3. Self-grooming

Although grooming is a comfort activity that cows are highly motivated to perform (McConnachie et al., 2018), brush use is a luxury activity, characterised by low behavioral resilience (Dawkins, 1990), and has been shown to decrease during disease (sub-clinical metritis: Mandel et al., 2017; lameness: Weigele et al., 2017; BRD: Toaff Rosenstein et al., 2016). A trade-off between brush location and the sensitivity of brush use for detecting stress and morbidity (Mandel et al., 2013; 2017) may help to explain why we failed to observe a significant difference between our two groups, or indeed a correlation between SAA and 'Brush'. The brush in the current study was readily accessible to all cows in the group with minimal effort, being located central to many resources including the feed barrier, a water trough and cubicles. Much variation exists in the reporting of self-grooming (licking) following illness and/or immune challenge in cows; studies report a decrease (LPS: Borderas et al., 2008; mastitis: Fogsgaard et al., 2012; BRD: Toaff-Rosenstein and Tucker, 2018), an increase (lameness: Almeida et al., 2008), and no di□erence (mastitis: Siivonen et al., 2011).

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

In the current study SCM cows performed more comfort behavior (including self-licking) than CTRL cows immediately following morning milking. This may be a response to mild udder discomfort or as a substitute for allogrooming (see section 4.2.3).

4.4.4. Environmental exploration

Des Roches et al. (2017; 2018) report behavioral changes (including reduced attentiveness) during the pre-clinical phase of an experimental mastitis model (prior to the upregulation of SCC and serum SAA), and during the acute phase (coinciding with raised levels of SCC and SAA), but not during the remission phase, even although high levels of SCC/SAA were still evident. This suggests that a peak in serum SAA corresponds with the remission, rather than the acute, phase of inflammation. The weak positive correlation between SAA and environmental exploration (‘ExpEnv’) described in the current study was unexpected since exploratory behavior would be predicted to decline with sickness/inflammation. If our test cohort contained a proportion of individuals in the pre-clinical phase (low salivary SAA but demonstrating sickness-lowered social exploration) and a proportion of individuals in the remission phase (high SAA with healthy baseline levels of social exploration) then this may offer an explanation. One of the behaviors included within the ‘ExpEnv’ measure was ‘explore sand’. Cows can spend a long-time sniffing sand prior to selection of a cubicle to lie down in. Although our SCM cows did take longer to lie down than the CTRL cows this difference failed to reach significance (data not shown). Ruminants generally display low baseline levels of exploratory behavior when maintained in intensive housing, since it is a largely unstimulating environment (De Rosa et al., 2009). Although a reduction in ‘ExpEnv’ was not evident in our SCM group overall (24h), the CTRL cows did display more interest in their surroundings than SCM cows during the hour following the morning milking, which coincided with the provision of the single daily meal. CTRL cows also displayed more exploratory behavior between 05:00 – 06:00h (the hour prior to morning milking/feed delivery), presumably in anticipation of what was to come. SCM cows, conversely, dominated exploratory behavior at quieter times of the day (00:00 – 01:00h and 13:00 – 14:00h), providing further evidence for a diurnal shift in activity.

4.5 Future Research

Using effect size statistics on the 24h data set, we classified the between-group differences in several measures as being biologically ‘inconclusive’; i.e., effect size differences between the treatment groups remain plausible but were not conclusive given our sample size, and these,

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

therefore, provide a promising focus for future research into behavioral correlates of subclinical disease states in cows. They included brush use, body push given, challenge given, head swipe given, head butt received and mutual head butt. On the basis of several unexpected correlations within the current study (significant, but occurring in the opposite direction than predicted), and that disease models have demonstrated that peak immunity (APP levels) occurs during remission and persist after the recovery of sickness behavior (des Roches et al., 2017, 2018), we recommend further studies to investigate the association between inflammatory markers and behavior over natural disease progression. In humans, sickness is characteristically accompanied by a feeling of 'malaise', an affective state that involves the negative subjective experience of depression, lethargy and anhedonia, and is induced by pro-inflammatory cytokines as part of the body's sickness response (Dantzer et al., 2004). Whether subclinically-mastitic cows similarly experience malaise or a 'malaise-like' affective state is not known (see Weary et al., 2009). However, it does seem possible based on the evidence of pro-inflammatory cytokine-induced anhedonia and depression-like states in rats (Dantzer et al., 2008), and therefore also merits further investigation.

5. CONCLUSIONS

By studying the behavior of a group of matched-pair cows over short distinct time periods (1h and 24h) we identified that sub-clinical mastitis (SCM) was associated with a reduction in activity, social exploration, the receipt of affiliative behavior, social reactivity (following the receipt of agonistic behavior), and an increase in the receipt of head swipes, compared to clinically healthy control (CTRL) cows. Several of these measures are low-resilience behaviors, which have previously been highlighted as having potential for early illness detection since they are expected to decrease earlier than core activities (Weary et al., 2009). Although no difference in any core maintenance behavior (feeding, drinking, lying duration) was detected, the SCM cows did demonstrate a preference for risk-adverse 'within-herd feeding', spending a greater proportion of time feeding in direct contact with two neighbours, and spending a lower proportion of their time feeding at the self-locking feed barriers than the CTRL cows. We present evidence for diurnal differences in the daily behavioral routine between the two groups, which indicates that SCM cows shift their activity to quieter times of the day. It seems likely that this is a tactic employed by the SCM cows to actively avoid agonistic encounters since the CTRL cows were more likely to perform and receive agonistic behavior. A positive relationship between SCC and SAA was observed, indicating salivary SAA (a marker of systemic inflammation) to be a potential physiological marker of

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

subclinical mastitis. The majority of our behavioral measures was also found to correlate with salivary SAA in a direction consistent with sickness behavior. Taken together, these findings demonstrate that physiological and behavioral changes associated with subclinical mastitis in cows are consistent with predictions for low-level sickness responses.

ACKNOWLEDGEMENTS

This study was funded by The John Oldacre Foundation. We would also like to thank the staff at Wyndhurst Farm for facilitating our research.

COMPETING INTERESTS STATEMENT

The authors have no competing interests to declare.

REFERENCES

AHDB, 2015. The Agriculture and horticulture development board dairy mobility scoresheet https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/Dairy%20Mobility%20Scoresheet_200427_WEB.pdf - accessed 14/07/20

Åkerstedt, M., K. P. Waller, and A. Sternesjö, 2007. Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples. J. Dairy Res. 74:198-203. https://doi.org/10.1017/S0022029906002305

Almeida, P. E., P. S. D. Weber, J. L. Burton, and A. J. Zanella, 2008. Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions. Domestic Anim. Endocrinol. 34(1):89-99. https://doi.org/10.1016/j.domaniend.2006.11.006

Andrew, W., J. Gao, S. M. Mullan, N. W. Campbell, A. Dowsey, and T. Burghardt, 2020. Visual identification of individual holstein friesian cattle via deep metric learning. Comput. Electron. Agric. https://arxiv.org/abs/2006.09205

Arakawa, H., K. Arakawa, and T. Deak, 2010. Sickness-related odor communication signals as determinants of social behavior in rat: a role for inflammatory processes. Horm. Behav. 57:30-341. https://doi.org/10.1016/j.yhbeh.2010.01.002

Barragan A. A., J. M. Piñeiro, G. M. Schuenemann, P. J. Rajala-Schultz, D. E. Sanders, J. Lakritz, and S. Bas, 2018. Assessment of daily activity patterns and biomarkers of pain, inflammation, and stress in lactating dairy cows diagnosed with clinical metritis. J. Dairy Sci. 101(9):8248-8258. https://doi.org/10.3168/jds.2018-14510

Benham, P. F. J., 1984. Social organisation in groups of cattle and the interrelationship between social and grazing behaviours under different grazing management systems. Doctoral dissertation, University of Reading, England.

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Bluthé, R. M., B. Michaud, K. W. Kelley, and R. Dantzer, 1996. Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. Neuroreport 7(9):1485-1488. https://doi.org/10.1097/00001756-199606170-00008

Borderas, T. F., A. M. Passillé, and J. Rushen, 2008. Behavior of dairy calves after a low dose of bacterial endotoxin. J. Anim. Sci. 86:2920–2927. https://doi.org/10.2527/jas.2008-0926

Bryant, P. A., J. Trinder, and N. Curtis, 2004. Sick and tired: Does sleep have a vital role in the immune system? Nature Rev. Immunol. 4:457-467. https://doi.org/10.1038/nri1369

Cerón, J. J., 2019. Acute phase proteins, saliva and education in laboratory science: an update and some reflections. BMC Vet. Res. 15**:**197. https://doi.org/10.1186/s12917-019-1931-8

Cohen, J., 1988. Chapter 6: Differences between proportions. Pages 184-185 in Statistical Power Analysis for the Behavioral Sciences, 2nd edition. Hillsdale, Lawrence Erlbaum Publishers.

Cyples, J. A., C. E. Fitzpatrick, K. E. Leslie, T. J. DeVries, D. B. Haley, and N. Chapinal, 2012. Short communication: The effects of experimentally induced *Escherichia coli* clinical mastitis on lying behavior of dairy cows. J. Dairy Sci. 95(5):2571-2575. https://doi.org/10.3168/jds.2011-5135.

Dantzer, R. 2004. Cytokine-induced sickness behaviour: A neuroimmune response to activation of innate immunity. Eur. J. Pharmacol. 500:399–411. https://doi.org/10.1016/j.ejphar.2004.07.040

Dantzer, R. and K. W. Kelley, 2007. Twenty years of research on cytokine-induced sickness behaviour. Brain Behav. Immun. 21(2):153-160. https://doi.org/10.1016/j.bbi.2006.09.006

Dantzer, R., J. C. O'Connor, G. G. Freund, R. W. Johnson, and K. W. Kelley, 2008. From inflammation to sickness and depression: When the immune system subjugates the brain. Nature Rev. Neurosci. 9(1):46-56. https://doi.org/10.1016/j.bbi.2006.09.006

Dawkins, M. S. 1990. From an animal's point of view: Motivation, fitness, and animal welfare. Behav. Brain Sci. 13:1–9. https://doi.org/10.1017/S0140525X00077104

De Rosa, G., F. Grasso, A. Braghieri, A. Bilancione, A. Di Francia, and F. Napolitano, 2009. Behavior and milk production of buffalo cows as affected by housing system. J. Dairy Sci. 92(3):907-912. https://doi.org/10.3168/jds.2008-1157

des Roches, A. D. B., M. Faure, A. Lussert, V. Herry, P. Rainard, D. Durand, and G. Foucras, 2017. Behavioral and patho-physiological response as possible signs of pain in dairy cows during *Escherichia coli* mastitis: A pilot study. J. Dairy Sci. 100:8385–8397. https://doi.org/10.3168/jds.2017-12796

des Roches, A. D. B., A. Lussert, M. Faure, V. Herry, P. Rainard, D. Durand, F. Wemelsfelder, and G. Foucras, 2018. Dairy cows under experimentally-induced *Escherichia coli* mastitis show negative emotional states assessed through Qualitative Behaviour

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Assessment. Appl. Anim. Behav. Sci. 206:1-11. https://doi.org/10.1016/j.applanim.2018.06.004

DeVries, T. J. and M. A. G. von Keyserlingk, 2005. Time of feed delivery affects the feeding and lying patterns of dairy cows. J. Dairy Sci. 88: 625–631. https://doi.org/10.3168/jds.S0022-0302(05)72726-0

DeVries, T. J., M. A. G. von Keyserlingk, and D. M. Weary, 2004. Effect of feeding space on the inter-cow distance, aggression, and feeding behavior of free-stall housed lactating dairy cows. J. Dairy Sci. 87:1432–1438. https://doi.org/10.3168/jds.S0022-0302(04)73293-2

Dollinger, J. and O. Kaufmann, 2013. Feeding behaviour in dairy cows with and without the influence of clinical diseases or subclinical disorders. Archiv. Tierzucht 56(14):149-159. https://doi.org/10.7482/0003-9438-56-014

Eckersall, P. D., F. J. Young, A. M. Nolan, C. H. Knight, C. McComb, M. M. Waterston, C. J. Hogarth, E. M. Scott, and J. L. Fitzpatrick, 2006. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. J. Dairy Sci. 89(5):1488-1501. https://doi.org/10.3168/jds.S0022-0302(06)72216-0

Endres, M. I., T. J. DeVries, M. A. G. von Keyserlingk, and D. M. Weary, 2005. Effect of feed barrier design on the behavior of loose housed lactating dairy cows. J. Dairy Sci. 88:2377–2380. https://doi.org/10.3168/jds.S0022-0302(05)72915-5

Fogsgaard, K. K., C. M. Røntved, P. Sørensen, and M. S. Herskin, 2012. Sickness behavior in dairy cows during *Escherichia coli* mastitis. J. Dairy Sci. 95(2):630–638. https://doi.org/10.3168/jds.2011-4350

Fogsgaard, K. K., T. W. Bennedsgaard, and M. S. Herskin, 2015. Behavioral changes in freestall-housed dairy cows with naturally occurring clinical mastitis. J. Dairy Sci. 98:1730–1738. https://doi.org/10.3168/jds.2014-8347

Fuller, G. and S. Allard, 2018. Preliminary data showing potential for salivary C-reactive protein as an indicator of welfare in western lowland gorillas (*Gorilla gorilla gorilla*). J. Vet. Behav. 28:58-62. https://doi.org/10.1016/j.jveb.2018.08.005

Galindo, F. and D. M. Broom, 2000. The relationships between social behaviour of dairy cows and the occurrence of lameness in three herds. Res. Vet. Sci. 69:75–79. https://doi.org/10.1053/rvsc.2000.0391

Galindo, F. and D. M. Broom, 2002. The effects of lameness on social and individual behavior of dairy cows. J. Appl. Anim. Welfare Sci. 5(3):193–201. https://doi.org/10.1207/S15327604JAWS0503_03

Gerlanc, D. and K. N. Kirby, 2012. bootES (Version 1.0). Retrieved from http://cran.r-project.org/web/packages/bootES/index.html

Gleerup, K. B., P. H. Andersen, L. Munksgaard, and B. Forkman, 2015. Pain evaluation in dairy cattle. Appl. Anim. Behav. Sci. 171:25-32. https://doi.org/10.1016/j.applanim.2015.08.023

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Goldhawk, C., N. Chapinal, D. M. Veira, D. M. Weary, and M. A. G. von Keyserlingk, 2009. Prepartum feeding behavior is an early indicator of subclinical ketosis. J. Dairy Sci. 92:4971–4977. https://doi.org/10.3168/jds.2009-2242

González, L. A., B. J. Tolkamp, M. P. Coffey, A. Ferret, and I. Kyriazakis, 2008. Changes in feeding behavior as possible indicators for the automatic monitoring of health disorders in dairy cows. J. Dairy Sci. 91:1017-1028. https://doi.org/10.3168/jds.2007-0530

Gröhn, Y. T., P. J. Rajala-Schultz, H. G. Allore, M. A. DeLorenzo, J. A. Hertl, and D. T. Galligan, 2003. Optimizing replacement of dairy cows: modeling the effects of diseases. Prev. Vet. Med. 61: 27-43. https://doi.org/10.1016/S0167-5877(03)00158-2

Gu, J. Q., Z. H. Wang, R. H. Gao, H. R. Wu, 2017. Cow behavior recognition based on image analysis and activities. Int. J. Agric. Biol. Eng. 10(3):165–174. https://doi.org/10.165-174.10.3965/j.ijabe.20171003.3080

Guzhva, O., H. Ardö, A. Herlin, M. Nilsson, K. Åström, and C. Bergsten, 2016. Feasibility study for the implementation of an automatic system for the detection of social interactions in the waiting area of automatic milking stations by using a video surveillance system. Comp. Elec. Agri. 127:506-509. https://doi.org/10.1016/j.compag.2016.07.010

Hart, B. L., 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12:123-137. https://doi.org/10.1016/S0149-7634(88)80004-6

Hart, B. L., 2010. Beyond fever. Pages 205-210 in Comparative Perspectives on Sickness Behavior. Encyclopedia of Animal Behavior. Elsevier.

Hedges, L.V., 1981. Distributional theory for Glass's estimator of effect size and related estimators. J. Educ. Stats 6:107–128. https://doi.org/10.3102%2F10769986006002107

Hoonhout, L., I. Reimert, and C. I. Daigle, 2017. Relationship between allogrooming and disease in feedlot steers: Social interactions may provide information about individual animal health. J. Anim. Sci. 95(s4):2. https://doi.org/10.2527/asasann.2017.003

Huzzey, J. M., T. J. DeVries, P. Valois, and M. A. G. von Keyserlingk, 2006. Stocking density and feed barrier design affect the feeding and social behavior of dairy cattle. J. Dairy Sci. 89:126–133. https://doi.org/10.3168/jds.S0022-0302(06)72075-6

Huzzey, J. M., D. M. Veira, D. M. Weary, and M. A. G. von Keyserlingk, 2007. Prepartum behavior and dry matter intake identify dairy cows at risk for metritis. J. Dairy Sci. 90:3220–3233. https://doi.org/10.3168/jds.2006-807

ISO 13366-1, 1997. Part 1: Microscopic method (Reference method). In: Milk – Enumeration of Somatic Cells. International Organization for Standardisation, Geneva, Switzerland. https://webstore.ansi.org/Standards/ISO/ISO133661997

Ito, K., D. M. Weary, and M. A. G. von Keyserlingk, 2009. Lying behavior: Assessing within- and between-herd variation in free-stall-housed dairy cows. J. Dairy Sci. 92:4412–4420. https://doi.org/10.3168/jds.2009-2235

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Ito, K., M. A. G. Von Keyserlingk, S. J. LeBlanc, and D. M. Weary, 2010. Lying behavior as an indicator of lameness in dairy cows. J. Dairy Sci. 93(8):3553-3560. https://doi.org/10.3168/jds.2009-2951

Jadhav, P. V., D. N. Das, K. P. Suresh, and B. R. Shome, 2018. Threshold somatic cell count for delineation of subclinical mastitis cases. Vet World 11(6):789-793. https://doi.org/10.14202/vetworld.2018.789-793.

Jensen, M. B. and K. L. Proudfoot, 2017. Effect of group size and health status on behavior and feed intake of multiparous dairy cows in early lactation. J. Dairy Sci. 100:9759–9768. https://doi.org/10.3168/jds.2017-13035

Jensen, M. B., L. Munksgaard, L.J. Pedersen, J. Ladewig, and L. Matthews, 2004. Prior deprivation and reward duration affect the demand function for rest in dairy heifers. Appl. Anim. Behav. Sci. 88:1–11. https://doi.org/10.1016/j.applanim.2004.02.019

Jensen, M. B., L. J. Pederson, and L. Munksgaard, 2005. The effect of reward duration on demand functions for rest in dairy heifers and lying requirements as measured by demand functions. Appl. Anim. Behav. Sci. 90:207–217. https://doi.org/10.1016/j.applanim.2004.08.006

Joshi, V., V. K. Gupta, A. G. Bhanuprakash, R. S. K. Mandal, U. Dimri, and Y. Ajith, 2018. Haptoglobin and serum amyloid A as putative biomarker candidates of naturally occurring bovine respiratory disease in dairy calves. Microb. Pathog. 116:33-37. https://doi.org/10.1016/j.micpath.2018.01.001

Kauppi, J. J., 2014. Dairy cow behaviour in relation to health, welfare and milking. Doctoral Dissertation, MTT Agrifood Research Finland and University of Helsinki, Faculty of Veterinary Medicine. https://core.ac.uk/reader/52252554

Kent, S., R. M. Bluthé, K. W. Kelley, and R. Dantzer, 1992. Sickness behavior as a new target for drug development. Trends in Pharmacol. Sci. 13:24-28. https://doi.org/10.1016/0165-6147(92)90012-U

Kester, H. J., D. E. Sorter, and J. S. Hogan, 2015. Activity and milk compositional changes following experimentally induced *Streptococcus uberis* bovine mastitis. J. Dairy Sci. 98(2):999-1004. https://doi.org/10.3168/jds.2014-8576

King, M. T. M., S. J. LeBlanc, E. A. Pajor, T. C. Wright, and T. J. DeVries, 2018. Behavior and productivity of cows milked in automated systems before diagnosis of health disorders in early lactation. J. Dairy Sci. 101:4343–4356. https://doi.org/10.3168/jds.2017-13686

Kirby, K. N. and D. Gerlanc, 2013. BootES: An R package for bootstrap confidence intervals on effect sizes. Behav. Res. 45:905–927. https://doi.org/10.3758/s13428-013-0330-5

Kováč, G., C. Tóthová, O. Nagy, O., and H. Seidel, 2011. Milk amyloid A and selected serum proteins in cows suffering from mastitis. Acta Vet. Brno 80:3–9. https://doi.org/10.2754/avb201180010003

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Kovačević-Filipović, M., V. Ilić, Z. Vujčić, B. Dojnov, M. Stevanov-Pavlović, Z. Mijačević, and T. Božić, 2012. Serum amyloid A isoforms in serum and milk from cows with *Staphylococcus aureus* subclinical mastitis. Vet. Immunol. Immunopathol. 145(1-2):120-128. https://doi.org/10.1016/j.vetimm.2011.10.015

Kume, S., K. Nonaka, T. Oshita, and T. Kozaka, 2010. Evaluation of drinking water intake, feed water intake and total water intake in dry and lactating cows fed silages. Livest. Sci. 128:46–51. https://doi.org/10.1016/j.livsci.2009.10.012

Kyriazakis, I. and B. J. Tolkamp, 2011. Hunger and Thirst. Pages 44–57 in Animal Welfare, Appleby, M.C., J. A. Mench, I. A. S. Olsson, and B. O. Hughes, Eds. 2nd ed. CAB International, Wallingford, UK.

Lecchi, C., F. Dilda, P. Sartorelli, and F. Ceciliani, 2012. Widespread expression of SAA and Hp RNA in bovine tissues after evaluation of suitable reference genes. Vet. Immunol. Immunopathol. 145:556-562. https://doi.org/10.1016/j.vetimm.2011.12.017

Lee, D. K., 2016. Alternatives to P value: confidence interval and effect size. Korean J. Anesthesiol. 69(6):555-562. https://doi.org/10.4097/kjae.2016.69.6.555

Littin, K., A. Acevedo, W. Browne, J. Edgar, M. Mendl, D. Owen, C. Sherwin, H. Würbel, and C. J. Nicol, 2008. Towards humane end points: Behavioural changes precede clinical signs of disease in a Huntington's disease model. Proc. Biol. Sci. 275:1865–1874. https://doi.org/10.1098/rspb.2008.0388

Llonch, P., E. Mainau, I. R. Ipharraguerre, F. Bargo, G. Tedó, M. Blanch, and X. Manteca, 2018. Chicken or the egg: The reciprocal association between feeding behavior and animal welfare and their impact on productivity in dairy cows. Front. Vet. Sci. 5:305. https://doi.org/10.3389/fvets.2018.00305

Lomb, J., H. W. Neave, D. M. Weary, S. J. LeBlanc, J. M. Huzzey, and M. A. G. von Keyserlingk, 2018. Changes in feeding, social, and lying behaviors in dairy cows with metritis following treatment with a nonsteroidal anti-inflammatory drug as adjunctive treatment to an antimicrobial. J. Dairy Sci. 101(5):4400-4411. https://doi.org/10.3168/jds.2017-13812

Lukas, J. M., J. K. Reneau, and J. G. Linn, 2008. Water intake and dry matter intake changes as a feeding management tool and indicator of health and estrus status in dairy cows. J. Dairy Sci. 91(9):3385-3394. https://doi.org/10.3168/jds.2007-0926

Mandel, R., H. R. Whay, C. J. Nicol, and E. Klement, 2013. The effect of food location, heat load, and intrusive medical procedures on brushing activity in dairy cows. J. Dairy Sci. 96:6506–6513. https://doi.org/10.3168/jds.2013-6941

Mandel, R., C. J. Nicol, H. R. Whay, and E. Klement, 2017. Short communication: Detection and monitoring of metritis in dairy cows using an automated grooming device. J. Dairy Sci. 100:5724-5728. https://doi.org/10.3168/jds.2016-12201

Manson, F. J. and M. C. Appleby, 1990. Spacing of dairy cows at a food trough. Appl. Anim. Behav. Sci. 26:69-81. https://doi.org/10.1016/0168-1591(90)90088-U

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Madouasse, A., J. N. Huxley, W. J. Browne, A. J. Bradley, and M. J. Green, 2010. Somatic cell count dynamics in a large sample of dairy herds in England and Wales. Prev. Vet. Med. 96:56–64. https://doi.org/10.1016/j.prevetmed.2010.05.005

McConnachie, E., A. M. C. Smid, A. J. Thompson, D. M. Weary, M. A. Gaworski, and M. A. G. Von Keyserlingk, 2018. Cows are highly motivated to access a grooming substrate. Biol. Lett. 14:1-4. https://doi.org/10.1098/rsbl.2018.0303

Medrano-Galarza, C., J. Gibbons, S. Wagner, A. M. de Passillé, and J. Rushen, 2012. Behavioral changes in dairy cows with mastitis, J. Dairy Sci. 95(12):6994-7002. https://doi.org/10.3168/jds.2011-5247

Munksgaard, L., M. B. Jensen, L. J. Pedersen, S. W. Hansen, and L. Matthews, 2005. Quantifying behavioural priorities - Effects of time constraints on behaviour of dairy cows, *Bos taurus*. Appl. Anim. Behav. Sci. 92:3–14. https://doi.org/10.1016/j.applanim.2004.11.005

Murata, H., N. Shimada, and M. Yoshioka, 2004. Current research on acute phase proteins in veterinary diagnosis: An overview. Vet. J. 168:28-40. https://doi.org/10.1016/s1090-0233(03)00119-9

Nakagawa, S., 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behav. Ecol. 15(6):1044–1045. https://doi.org/10.1093/beheco/arh107

Nakagawa, S. and I. C. Cuthill, 2007. Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol. Rev. 82:591–605. https://doi.org/10.1111/j.1469-185x.2007.00027.x

Nakagawa, S. and T. M. Foster, 2004. The case against retrospective statistical power analyses with an introduction to power analysis, Acta Ethol. 7:103–108. https://doi.org/10.1007/s10211-004-0095-z

Napolitano, F., U. Knierim, F. Grasso, and G. De Rosa, 2009. Positive indicators of cattle welfare and their applicability to on-farm protocols. Ital. J. Anim. Sci. 8 (s1):355-365. https://doi.org/10.4081/ijas.2009.s1.355

Nazifi, S., A. Khoshvaghti, and H. R. Gheisari, 2008. Evaluation of serum and milk amyloid A in some inflammatory diseases of cattle. Iranian J. Vet. Res. 9(3):222-226.

Nazifi, S., M. Haghkhah, Z. Asadi, M. Ansari-Lari, M. R. Tabandeh, Z. Esmailnezhad, and M. Aghamir, 2011. Evaluation of sialic acid and acute phase proteins (haptoglobin and serum amyloid A) in clinical and subclinical bovine mastitis. Pakistan Vet. J. 31(1):55-59.

Neave H. W., J. Lomb, D. M. Weary, S. J. Leblanc, J. M. Huzzey, and M. A. G. Von Keyserlingk, 2018. Behavioral changes before metritis diagnosis in dairy cows. J. Dairy Sci. 101(5):4388-4399. https://doi.org/10.3168/jds.2017-13078

O'Mahony, M. C., A. M. Healy, D. Harte, K. G. Walshe, P. R. Torgerson, and M. L. Doherty, 2006. Milk amyloid A: Correlation with cellular indices of mammary inflammation

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

in cows with normal and raised serum amyloid A. Res. Vet. Sci. 80:155–161. https://doi.org/10.1016/j.rvsc.2005.05.005

Opp, M. R., 2005. Cytokines and sleep. Sleep Med. Rev. 9(5):355-364. https://doi.org/10.1016/j.smrv.2005.01.002

Patbandha, T. K., T. K. Mohanty, S. S. Layek, A. Kumaresan, and K. Behera, 2012. Application of pre-partum feeding and social behaviour in predicting risk of developing metritis in cross bred cows. Appl. Anim. Behav. Sci. 139:10-17. https://doi.org/10.1016/j.applanim.2012.03.014

Prickett, J. and J. Zimmerman, 2010. The development of oral fluid-based diagnostics and applications in veterinary medicine. Animal Health Res. Rev. 11:1–10. https://doi.org/10.1017/S1466252310000010

Proudfoot, K. L., J. M. Huzzey, and M. A. G. von Keyserlingk, 2009. The effect of dystocia on the dry matter intake and behavior of Holstein cows. J. Dairy Sci. 92:4937–4944. https://doi.org/10.3168/jds.2009-2135

Pyörälä, S., M. Hovinen, H. Simojoki, J. Fitzpatrick, P. D. Eckersall, and T. Orro, 2011. Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. Vet. Rec. 168(20):535. https://doi.org/10.1136/vr.d1120

Rahman, M., U. Müller, H. Sauerwein, P. Heimberg, and F. Ceciliani, 2013. Investigation of salivary acute phase proteins in calves. Pages 213-216 in Farm Animal Proteomics 2013, de Almeida A. et al. (eds). Wageningen Academic Publishers, Wageningen.

Sato, S., S. Sako, and A. Maeda, 1991. Social licking patterns in cattle (*Bos taurus*): influence of environmental and social factors. Appl. Anim. Behav. Sci. 32:3-12. https://doi.org/10.1016/S0168-1591(05)80158-3

Sato, S., K. Tarumizu, and K. Hatae, 1993. The influence of social factors on allogrooming in cows. Appl. Anim. Behav. Sci. 38:235-244. https://doi.org/10.1016/0168-1591(93)90022-H

Schirmann, K., D. M. Weary, W. Heuwieser, N. Chapinal, R. L. A. Cerri, and M. A. G. von Keyserlingk, 2016. Short communication: Rumination and feeding behaviors differ between healthy and sick dairy cows during the transition period. J. Dairy Sci. 99:9917–9924. https://doi.org/10.3168/jds.2015-10548

Sepúlveda-Varas, P., D. M. Weary, and M. A. G. von Keyserlingk, 2014. Lying behavior and postpartum health status in grazing dairy cows. J. Dairy Sci. 97:6334–6343. https://doi.org/10.3168/jds.2014-8357

Sepúlveda-Varasa, P., K. L. Proudfoot, D. M. Weary, and M. A. G. Keyserlingk, 2016. Changes in behaviour of dairy cows with clinical mastitis. Appl. Anim. Behav. Sci. 175:8-13. https://doi.org/10.1016/j.applanim.2014.09.022

Siivonen, J., S. Taponen, M. Hovinen, M. Pastell, B. Joop Lensink, S. Pyörälä, and L. Hanninen, 2011. Impact of acute clinical mastitis on cow behaviour. Appl. Anim. Behav. Sci. 132:101–106. https://doi.org/10.1016/j.applanim.2011.04.005

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Sordillo, L. M., K. Shafer-Weaver, and D. DeRosa, 1997. Immunobiology of the mammary gland. J. Dairy Sci. 80:1851–1865. https://doi.org/10.3168/jds.S0022-0302(97)76121-6

Stangaferro, M. L., R. Wijma, L. S. Caixeta, M. A. Al-Abri, and J. O. Giordano, 2016. Use of rumination and activity monitoring for the identification of dairy cows with health disorders. Part II. Mastitis. J. Dairy Sci. 99:7411–7421. https://doi.org/10.3168/jds.2016-10907

Steensels, M., E. Maltz, C. Bahr, D. Berckmans, A. Antler, and I. Halachmi, 2017. Towards practical application of sensors for monitoring animal health: the effect of post-calving health problems on rumination duration, activity and milk yield. J. Dairy Res. 84:132–138. https://doi.org/10.1017/S0022029917000176

Ternman, E., L. Hänninen, M. Pastell, S. Agenäs, and P. Nielsen, 2013. Sleep in dairy cows recorded with a non-invasive EEG technique. Appl. Anim. Behav. Sci. 140:25-32. https://doi.org/10.1016/j.applanim.2012.05.005

Tizard, L., 2008. Sickness behavior, its mechanisms and significance. Anim. Health Res. Rev. 9:87-99. https://doi.org/10.1017/S1466252308001448

Toa□-Rosenstein, R. L. and C. B. Tucker, 2018. The sickness response at and before clinical diagnosis of spontaneous bovine respiratory disease. Appl. Anim. Behav. Sci. 201:85-92. https://doi.org/10.1016/j.applanim.2018.01.002

- Toaff-Rosenstein, R. L., L. J. Gershwin, A. J. Zanella, and C. B. Tucker, 2016. The sickness response in steers with induced bovine respiratory disease before and after treatment with a non-steroidal anti-inflammatory drug. Appl. Anim. Behav. Sci. 181:49-62. https://doi.org/10.1016/j.applanim.2016.05.016

Val-Laillet, D., A. M. de Passille, J. Rushen, and M. A. G. von Keyserlingk, 2008. The concept of social dominance and the social distribution of feeding-related displacements between cows. Appl. Anim. Behav. Sci. 111:158–172. https://doi.org/10.1016/j.applanim.2007.06.001

Veissier, I., P. Le Neindre, and G. Trillat, 1989. The use of circadian behaviour to measure adaptation of calves to changes in their environment. Appl. Anim. Behav. Sci. 22:1-12. https://doi.org/10.1016/0168-1591(89)90075-0

Veissier I., M. M. Mialon, and K. H. Sloth, 2017. Short communication: Early modification of the circadian organization of cow activity in relation to disease or estrus. J. Dairy Sci. 100:3969–3974. https://doi.org/10.3168/jds.2016-11853

Verbeke, J., S. Piepers, K. Supré, and S. De Vliegher, 2014. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. J. Dairy Sci. 97:6926–6934. https://doi.org/10.3168/jds.2016-11853

von Keyserlingk, M.A.G. and D. M. Weary, 2010. Review: Feeding behaviour of dairy cattle: Measures and applications. Can. J. Anim. Sci. 90(3):303-309. https://doi.org/10.4141/CJAS09127

UOB Open

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Wagner, N., V. Antoine, M. M. Mialon, R. Lardy, M. Silberberg, J. Koko, and I. Veissier, 2020. Machine learning to detect behavioural anomalies in dairy cows under subacute ruminal acidosis. Comput. Electron. Agric. 170:105233. https://doi.org/10.1016/j.compag.2020.105233

Weary, D. M., L. Niel, F. C. Flower, and D. Fraser, 2006. Identifying and preventing pain in animals. Appl. Anim. Behav. Sci. 100(1-2):64-76. https://doi.org/10.1016/j.applanim.2006.04.013

Weary, D. M., J. M. Huzzey, and M. A. G. von Keyserlingk, 2009. Using behavior to predict and identify ill health in animals. J. Anim. Sci. 87:770–777. https://doi.org/10.2527/jas.2008-1297

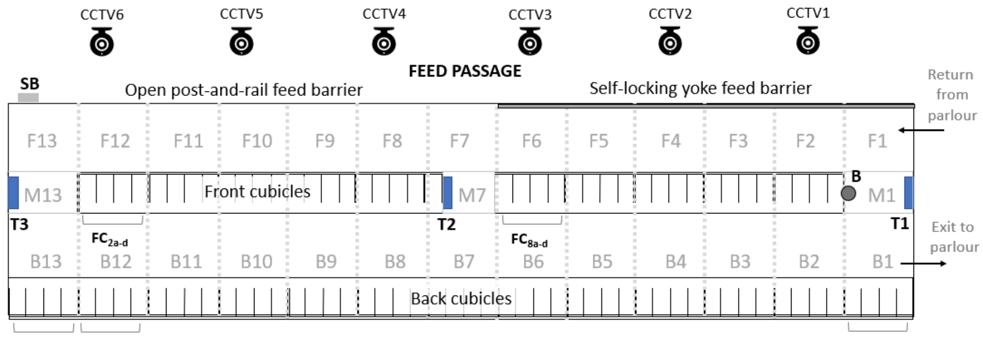
Weigele, H. C., L. Gygax, A. Steiner, B. Wechsler, and J. B. Burla, 2017. Moderate lameness leads to marked behavioral changes in dairy cows. J. Dairy Sci. 101:2370–2382. https://doi.org/10.3168/jds.2017-13120

Yeiser, E. E., K. E. Leslie, M. L. McGilliard, and C. S. Petersson-Wolfe, 2012. The effects of experimentally induced *Escherichia coli* mastitis and flunixin meglumine administration on activity measures, feed intake, and milk parameters. J. Dairy Sci. 95:4939–4949. https://doi.org/10.3168/jds.2011-5064

UOB Open

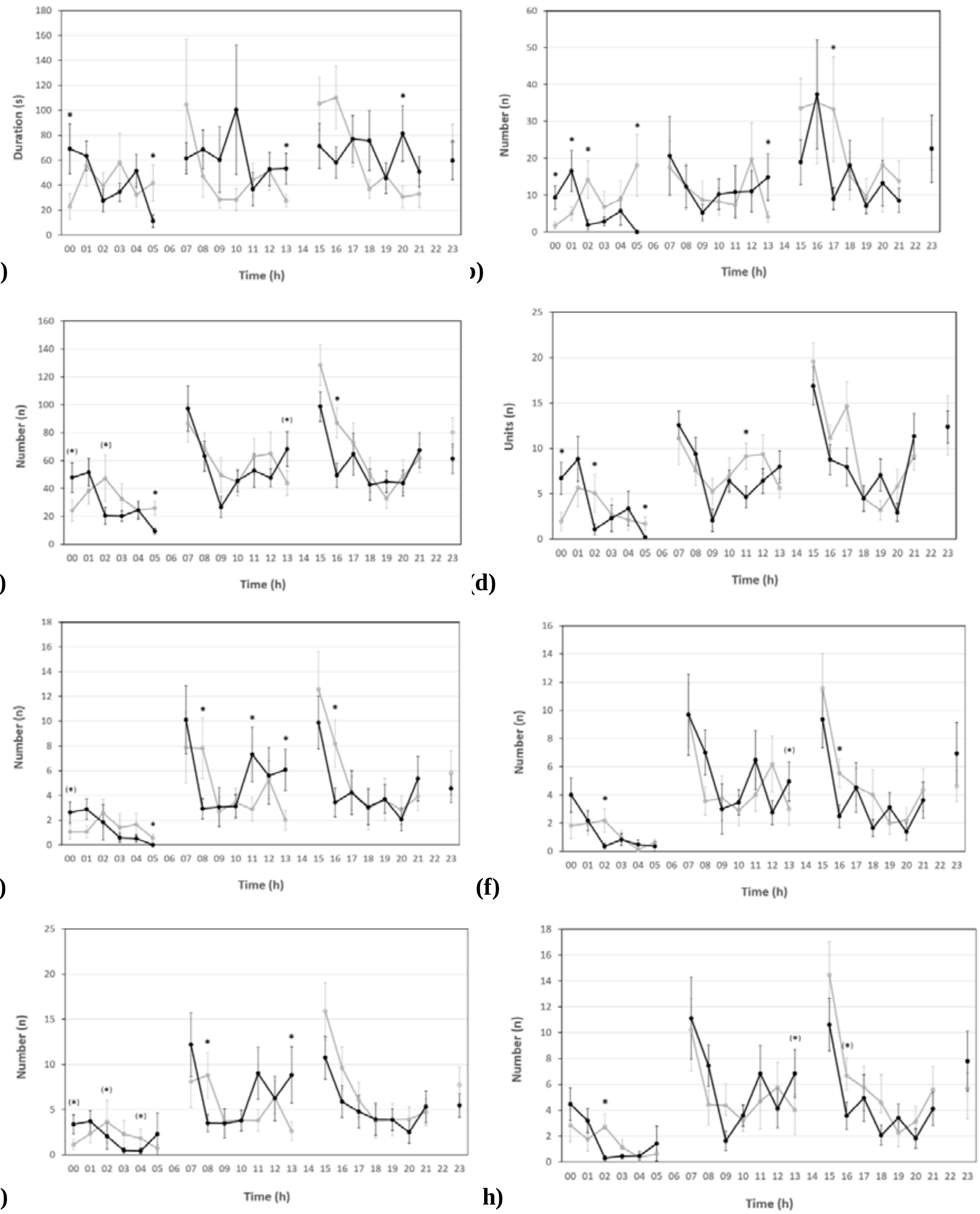
bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 1: Plan of the home pen including CCTV camera position and virtual division of floor-space (F1-13, M1/7/13, B1-13) for logging cow position. SB = salt bin, T1-3 = water troughs, B = brush



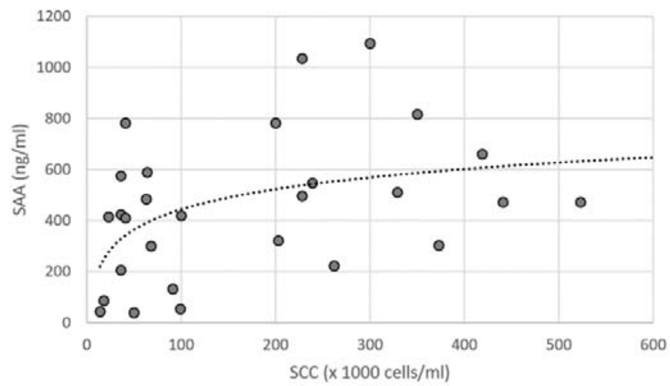
bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 2: Diurnal differences in dairy cow behavior (grey = CTRL, black = SCM): (a) explore environment, 'ExpEnv', (b) explore social, 'ExpSoc', (c) behavioral transitions, 'Trans', (d) distance moved, 'Dist', (e) agonistic behavior given, 'FocSocG', (f) agonistic behavior received, 'FocSocR', (g) all social behavior given, 'SocG', (h) all social behavior received, 'SocR'. An asterisk in brackets '(*)' denotes a statistical significance (P < 0.10) between the two groups based upon a Wilcoxon SR Test alone; an asterisk '*' denotes that the difference was confirmed by effect size statistics (Hedges' g). Milking times: 06:00 - 07:00h, 14:00 - 15:00h, 22:00 - 23:00h



bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 3: Relationship between somatic cell count (SCC) in milk and salivary serum amylase-A (SAA) in dairy cattle (n = 28)



bioRxiv preprint doi: https://doi.org/10.1101/2021.01.10.426092; this version posted January 10, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure 4: Relationship between somatic cell count (SCC) and two measures of activity (grey = CTRL, black = SCM): (a) behavioral transitions ('Trans'); (b) distance covered ('Dist').

(a) **(b)**